Major changes in phosphorylation of chromatin-associated non-histone proteins accompany development in the slime mould *Dictyostelium discoideum*

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Striking changes in protein and RNA synthesis were shown to accompany development in the slime mould *Dictyostelium discoideum*. These changes are, at least in part, directly attributable to control at the level of transcription. Analysis of nuclear proteins and their states of phosphorylation by two-dimensional gel electrophoresis and autoradiography showed only minor changes in the species of proteins detectable by Coomassie Blue staining during the vegetative growth and development phases. Major changes, however, were detected in their patterns of phosphorylation, with major differences from the vegetative pattern occurring during both early development (0-2h) and late development (8-12h). These changes coincide with major changes in polypeptide synthesis and in nuclear and cytoplasmic RNA complexity.

The cellular slime mould *Dictyostelium discoideum* is an ideal eukaryotic system in which to analyse changes at the molecular and biochemical levels associated with differentiation. Growing cells exist as undifferentiated unicellular amoebae dividing by binary fission. However, on starvation, amoebae go through a synchronous series of morphological and biochemical changes resulting in the formation of a multicellular fruiting body, consisting of two major differentiated cell types: spore cells and stalk cells.

During this developmental programme, changes in the rates of synthesis and accumulation of many species of protein and RNA (Tuchman *et al.*, 1974; Firtel, 1972) occur. Although some of these changes may be related to changes in RNA stability, it is believed that to a large extent the expression of developmentally regulated genes is controlled at the level of transcription (Alton & Lodish, 1977a,b; Williams *et al.*, 1979; Landfear *et al.*, 1982).

Analysis of the proteins synthesized by high-resolution two-dimensional gel electrophoresis (Alton & Lodish, 1977a) and analysis of certain cloned mRNA species by RNA-DNA hybridization (Blumberg & Lodish, 1980a) have shown that, whereas changes in the amounts of mRNA

species and the rates of synthesis of proteins occur throughout development, most of the transcription of new genes appears to occur during late aggregation. RNA-excess hybridization has shown that at this stage of development transcription of new RNA species corresponding to expression of 26% of the single-copy portion of the genome occurs (Blumberg & Lodish, 1980a,b).

Numerous investigations support the general notion that factors effecting such transcriptional control of gene expression may reside in the non-histone-protein fraction of the nucleus. For instance, studies *in vitro* have implicated non-histone protein in the tissue-specific restriction of chromatin (Gilmour, 1980) and control of the expression of specific genes (Tsai *et al.*, 1976; Kuehn *et al.*, 1979) as well as conferring transcriptional activity on chromatin (Weisbrod & Weintraub, 1979).

In particular, changes in the phosphorylation of eukaryotic nuclear proteins have been shown to accompany major changes in cell function and structure, as a result of growth, development, hormonal stimulation and malignant transformation (Kleinsmith, 1975). Hence it is reasonable to assume that phosphorylation and dephosphorylation of nuclear non-histone proteins probably play some role in the control of gene expression. We describe here an analysis of nuclear proteins and their states of phosphorylation during development in the cellular slime mould *D. discoideum*, the results of which may help lead to a

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better understanding of the function of nuclear proteins and their possible role in the regulation of growth and differentiation in this lower eukaryote.

Materials and methods

Chemicals

As far as possible all chemicals were of analytical grade and were obtained mainly from British Drug Houses (Poole, Dorset, U.K.). Bacteriological peptone and yeast extract were purchased from Oxoid (London E.C.4, U.K.). Analytical-grade urea was prepared as an 8M stock solution and deionized immediately before use through a column of AG 501-X8 (D) mixed-bed resin (Bio-Rad Laboratories, Bromley, Kent, U.K.). Ampholine carrier ampholytes (40%; pH range 3.5–10) were obtained from LKB Instruments (South Croydon, Surrey, U.K.). Mes, *p*-chloromercuribenzoic acid (a cysteine proteinase inhibitor), dithiothreitol, guanidine hydrochloride (type 1), bovine serum albumin, cytochrome *c* and ferritin were all obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Coomassie Brilliant Blue R and Carbowax (M_r 15000–20000) were purchased from G. T. Gurr (High Wycombe, Bucks., U.K.). Carrier-free [32 P]P_i was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Phenylmethanesulphonyl fluoride (a serine proteinase inhibitor) was purchased from Calbiochem (La Jolla, CA, U.S.A.).

Cell culture

Strain Ax-2 of *Dictyostelium discoideum* was grown at 22°C on rotary shakers at 160 rev./min as described by Watts & Ashworth (1970). Typically cells had a doubling time of 10h.

Myxamoebae were allowed to develop on Millipore filters as described by Alton & Lodish (1977a). Development was carried out at 22°C in the dark, in a humid environment.

Labelling conditions

Carrier-free [32 P]P_i was added directly to vegetative cells at a concentration of 2.6 mCi/700 ml of culture up to 12h before harvesting.

Cells were labelled during development as described by Delaney *et al.* (1983). For radioisotopic labelling of vegetative and developing cells, equivalent isotopic concentrations of [32 P]P_i were used. Developing myxamoebae were labelled from the beginning of development and harvested at 2, 4, 8 and 12h of the developmental programme in order to analyse the cumulative changes in phosphorylation of nuclear proteins.

Under these conditions, morphological development proceeded normally and fruiting bodies were formed after approx. 24h.

Preparation of nuclei

All operations were carried out at 4°C unless otherwise stated. Nuclei were isolated by a modification of the method of Rickwood & Osman (1979) as described previously. Total cell lysis was checked by phase-contrast microscopy. The nuclear pellet was then washed twice in lysis buffer (Rickwood & Osman, 1979), and the cell cytoplasm was dialysed against 8M-urea/20mM-Tris/HCl (pH 7.5).

Preparation of chromatin and nuclear protein fractions

Nuclei were resuspended in 20 vol. of 0.14M-NaCl/5mM-EDTA/0.05M-Tris/HCl (pH 7.5) containing 1mM-phenylmethanesulphonyl fluoride and 1mM-*p*-chloromercuribenzoate and saline-soluble proteins and chromatin were isolated as described by Rickwood & MacGillivray (1975). The saline-soluble fraction was dialysed against 8M-urea/20mM-Tris/HCl (pH 7.5), and the pellet of chromatin was solubilized by suspension in 8M-urea/20mM-Tris/HCl (pH 7.5).

Gel electrophoresis

Removal of nucleic acids from samples. Before isoelectric focusing, nucleic acids, which would interfere with focusing (O'Farrell, 1975), were removed by centrifugation in urea/Cs₂SO₄ gradients as described by Adameitz & Hiltz (1976). Gradients were unloaded by upward displacement with Maxidens (Nyegaard and Co. A/S, Oslo, Norway) and the proteins were recovered in the top 1 ml of the gradient, devoid of all but low- M_r nucleotides. Protein fractions were dialysed against 8M-urea/20mM-Tris/HCl (pH 7.5) containing 1mM-dithiothreitol.

The 32 P specific radioactivity per μ g of protein of each fraction was measured by the protein assay of Schaffner & Weissmann (1973) and liquid-scintillation counting of hot-acid-precipitable 32 P radioactivity, which has been shown to be protein-associated as alkali-labile phosphodiester bonds (Cordingley, 1980).

No loss of protein-associated phosphate was observed during the isolation and preparation of protein fractions, as analysed by the specific radioactivity of total protein samples immediately on isolation and after preparation for electrophoresis (results not shown).

Two-dimensional-electrophoretic separation of nuclear proteins

After removal of nucleic acids from samples, proteins were analysed by two-dimensional gel electrophoresis, involving isoelectric focusing in the first dimension followed by separation in the

presence of sodium dodecyl sulphate in the second dimension, as previously described (Sinclair & Rickwood, 1981).

Analysis of gels

After electrophoresis, the two-dimensional gels were gently removed from the glass plates, soaked in 7% (w/v) trichloroacetic acid for 30 min and heated at 90°C to hydrolyse any remaining low- M_r polynucleotides. After cooling, the gels were stained with 0.1% (w/v) Coomassie Blue, and then photographed and autoradiographed as previously described (Sinclair & Rickwood, 1981). Exposure times for gels were such that the product of the time and radioactivity was constant for all gels where possible. This form of analysis of cumulative changes in phosphorylation of proteins *in vivo* allows only a direct comparison of the presence or absence of phosphorylated species of proteins during vegetative growth and development. The changes in phosphate pool size, which are known to accompany development in *D. discoideum* (Gezelius *et al.*, 1973), make direct comparisons of the extent of phosphorylation of specific proteins invalid.

The results presented here were common to at least three separate experiments carried out over the course of 2–3 months. Minor changes in proteins and their phosphorylation not common to at least three of the experiments have been deemed unreproducible and have not been discussed.

Results

Saline-soluble proteins

As with higher-eukaryotic nuclei, a significant proportion (approx. 66%) of the nuclear proteins were removed by extraction with 0.14M-NaCl/5mM-EDTA/50mM-Tris/HCl (pH 7.4); the bulk of these proteins are associated with rRNA and heterogeneous nuclear RNA present in the nucleus (Rickwood & Birnie, 1976; Samarina *et al.*, 1968; Firtel & Pederson, 1975). The chromatin was insoluble in this medium, and the extractions did not solubilize detectable amounts of DNA. Analysis of the extracted proteins revealed that, as with other species (Tuchman *et al.*, 1974; Comings & Tack, 1973), many of the proteins extracted by 0.14M-NaCl from the chromatin were similar to those that remained associated with the DNA (cf. Figs. 1 and 5). However, some significant differences, especially in the more minor Coomassie-Blue-stained species, could be observed (e.g. spots 12, 13 and 14 of Fig. 1a). This suggests that at least some degree of specificity of polypeptides occurs in this fraction. Autoradiography (Fig. 1b) showed that, although a few of the major species of phos-

phorylated proteins of this fraction were similar to those seen in the chromosomal fraction (Fig. 5b), in general the phosphorylation pattern of the saline-soluble fraction was dissimilar to that of the chromosomal fraction, suggesting that the state of phosphorylation of proteins in these fractions is different from each other, and both fractions are also very different from the cytoplasmic phosphorylated species (see Fig. 4a).

Analysis, by Coomassie Blue staining of two-dimensional gels, of proteins extracted from purified nuclei isolated during vegetative growth and various stages of development by homogenization in saline/EDTA/Tris is shown in Fig. 2. The major polypeptides of the nuclear saline-soluble fraction were generally similar, both quantitatively and qualitatively, during vegetative growth and throughout development. However, differences from the vegetative pattern were observable during development in some of the more minor species. For instances, at 2h of development (Fig. 2b), the overall Coomassie Blue-stained pattern became more complex, with increases in the number of high- M_r acidic and basic species of polypeptide. This more complex pattern was maintained at 4h and 8h (results not shown) of development, with only a very few minor quantitative changes in polypeptides occurring between gels. At 12h of development, however (Fig. 2c), differences from those patterns at 2–8h of development were seen in that many of the high- M_r acidic and basic species, which appeared at 2h of development and persisted through until 8h, disappeared.

Analysis of the cumulative changes in phosphorylation of these proteins by autoradiography of the gels (Fig. 3) showed that the patterns of phosphorylation of the saline-soluble proteins isolated from nuclei of vegetative amoebae (Fig. 3a) were qualitatively and quantitatively quite different from those seen at 2h (Fig. 3b). The major differences occurred in the acidic low- M_r and basic high- M_r areas of the autoradiograms. However, few differences in the phosphorylation patterns of these saline-soluble proteins were observed between the 2h and 4h or 8h developmental time stages (results not shown). However, at 12h of development (Fig. 3c), major changes in the autoradiographic pattern occurred, with a major increase in intermediate- M_r acidic species (spots 3'–6' of Fig. 3c) and a total loss of others (e.g. spots 7' and 9' of Figs. 3a and 3b). At least some of these phosphorylated species which changed during development co-migrated with Coomassie-Blue-stained species which themselves did not vary during development (e.g. Coomassie Blue stained spots 3 and 8 of Figs. 2a and 2b; with autoradiogram spots 7' and 8' of Figs. 3a and 3b).

Fig. 1. *NaCl* (0.14M)-soluble nuclear proteins of vegetative amoebae

The 0.14M-*NaCl*-soluble nuclear proteins isolated from amoebae labelled with [32 P] P_i during vegetative growth were separated by two-dimensional gel electrophoresis, involving isoelectric focusing (IEF) in the first dimension followed by separation in the presence of sodium dodecyl sulphate (SDS) in the second dimension. The gel was stained with Coomassie Blue (a) and autoradiographed (b).

Fig. 2. *Changes in 0.14M-*NaCl*-soluble nuclear proteins during development*

The 0.14M-*NaCl*-soluble nuclear proteins isolated from amoebae labelled with [32 P] P_i during vegetative growth (a) and at 2h (b) and 12h (c) of development were separated by two-dimensional gel electrophoresis. Gels were stained with Coomassie Blue.

Fig. 3. Changes in phosphorylation of 0.14M-NaCl-soluble nuclear proteins during development. Corresponding autoradiograms of the two-dimensional gels of the 0.14M-NaCl-soluble nuclear proteins shown in Fig. 2 during vegetative growth (a) and at 2h (b) and 12h (c) of development.

The changes observed in both the Coomassie-Blue-stained patterns and the autoradiographs were not the result of cytoplasmic contamination, since the phosphorylated and non-phosphorylated species present in the cytoplasm were distinctly different from the saline-soluble species. Similarly the changes in phosphorylation of proteins of the cytoplasm during the development were totally different from those changes in phosphorylation observed in the saline-soluble fraction (Fig. 4).

Total chromosomal proteins

The proteins associated with the DNA after extraction with 0.14M-NaCl/5mM-EDTA/50mM-Tris/HCl (pH 7.4) were complex, with M_r values between 10000 and 200000 and isoelectric points between approx. pH 3.5 and 9.0 (Fig. 5a). Both phosphorylated and non-phosphorylated species were present in this fraction. The protein that migrated on two-dimensional gels in the expected position of actin (Alton & Lodish, 1977a) could be identified (spot 1), and this co-migrated with a minor phosphorylated species observed in the

corresponding autoradiogram of the fraction (spot 10' of Fig. 5b).

However, as with the saline-soluble fraction, most of the major proteins were not extensively phosphorylated, and most phosphorylated species were not associated with Coomassie-Blue-stained polypeptides.

Analysis of total chromatin-associated proteins by Coomassie Blue staining of two-dimensional gels showed no reproducible differences between chromatin-associated proteins isolated during vegetative growth (Fig. 5a) or isolated from amoebae at 2h, 4h, 8h or 12h of development (results not shown).

In contrast with the Coomassie Blue staining, analysis of the cumulative changes in phosphorylation of these chromosomal proteins, by autoradiography of the two-dimensional gels, did show differences in phosphorylation between those chromatin-associated proteins isolated from amoebae during vegetative growth (Fig. 5) and development (Figs. 6a–6e) and between those isolated from amoebae during various stages of

Fig. 4. *Changes in phosphorylation of cytoplasmic proteins during development*

Cytoplasmic proteins isolated from amoebae labelled with [^{32}P]P_i during vegetative growth (a) and at 2 h (b) and 12 h (c) of development were separated by two-dimensional gel electrophoresis, and the gels were autoradiographed as described in the Materials and methods section.

Fig. 5. *Chromatin-associated proteins of vegetative amoebae*

Chromatin-associated proteins isolated from amoebae labelled with [^{32}P]P_i during vegetative growth were separated by two-dimensional gel electrophoresis. The gel was stained with Coomassie Blue (a) and autoradiographed (b).

development. For instance, at 2 h of development (Fig. 6b), some phosphorylated species seen during vegetative growth (e.g. spots 12', 15' and 16' of Fig. 6a) were no longer present, whereas other, new, low- M_r acidic phosphorylated polypeptides not present during vegetative growth appeared (e.g. spots 7', 9', 19' and 20'). At 4 h of development (Fig. 6c) the phosphorylation pattern was similar to that observed at 2 h of development, except for slight

differences in the presence or absence of some of the more minor phosphorylated species (cf. Figs. 6b and 6c). Similarly, at 8 h of development (Fig. 6d) changes in the presence of minor species of phosphorylated proteins were also seen, though two major differences observed were the virtual disappearance of a major phosphorylated species observed during vegetative growth (Fig. 6a) and at 2–4 h (Figs. 6b and 6c) of development (spot 18') and the appearance of a phosphorylated species which co-migrated with the major Coomassie-Blue-stained actin (spot 1) also seen during vegetative growth (Fig. 6a). At 12 h of development (Fig. 6e) major differences were observed from the vegetative and earlier developmental time points, in that there was a total loss of the major acidic phosphorylated species observed earlier, and this was accompanied by a large increase in the complexity of basic phosphorylated species. Unlike the saline-soluble fraction, none of the phosphorylated species observed to vary during development co-migrated with any Coomassie-Blue-stained species, and because of the lack of co-migration between Coomassie-Blue-stained spots and autoradiograph spots it is impossible to tell whether these changes were due to the absence or presence of phosphorylated species or simply the changes in state of phosphorylation of proteins undetected by Coomassie Blue staining.

Discussion

We have shown that the nucleus and cytoplasm of *D. discoideum* contain different types of phosphoproteins; further, the nuclear phosphoproteins can be differentially extracted by saline solutions. Previous work has shown that *D. discoideum* contains a major cyclic AMP-dependent protein kinase (de Gunzburg *et al.*, 1984) and it is likely that, as in other cells, this kinase has different protein substrates *in vivo*. Much evidence has accumulated which suggests that in a wide range of organisms phosphoproteins are important in the regulation of gene transcription (Kleinsmith, 1975). The present study was undertaken to see if similar correlations between the phosphorylation of nuclear proteins were present also in *D. discoideum* during differentiation.

The nuclear proteins extracted by NaCl/Tris/EDTA solutions, the saline-soluble proteins, include the soluble nucleoplasmic proteins and loosely bound chromatin proteins, but it is accepted that most of these proteins are derived from ribosomes and their precursors as well as informosome-like particles (heterogeneous nuclear ribonucleoprotein) (Samarina *et al.*, 1968; Firtel & Pederson, 1975; Rickwood & Birnie, 1976). In mammalian cells there is some evidence that the

Fig. 6. Changes in phosphorylation of chromatin-associated non-histone proteins during development

Chromatin-associated non-histone proteins isolated from amoebae labelled with [32 P]P_i during vegetative growth (a) and at 2 h (b), 4 h (c), 8 h (d) and 12 h (e) of development were separated by two-dimensional gel electrophoresis, and the gels were autoradiographed as described in the Materials and methods section.

species of protein associated with the hnRNA is tissue-specific (Pederson, 1974). Similarly, in *D. discoideum* it appears that some changes take place in the species of heterogeneous nuclear ribonucleoprotein during development (Firtel & Pederson, 1975). One notable feature of development in *D. discoideum* is the extensive degradation of existing ribosomes, followed by the synthesis of new ribosomes (Cocucci & Sussman, 1970), and, although the newly synthesized rRNA appears to be identical with the existing ribosomes' rRNA (Batts-Young *et al.*, 1980), significant differences are found in the proteins associated with the rRNA (Ragmagopol & Ennis, 1981). Hence the changes observed in both the Coomassie-Blue-stained proteins and the phosphorylated proteins probably reflect changes occurring in the proteins associated with RNA in the nucleus.

A number of the polypeptides extracted by NaCl/Tris/EDTA co-migrate with some of the polypeptides associated with chromatin, and indeed a minor fraction of them may be the result of cross-contamination between the two fractions. However, clear evidence of major differences between the two fractions is provided by the different response seen in both the Coomassie Blue staining and autoradiography of the saline-soluble fraction when compared with the Coomassie Blue staining and autoradiography of the chromatin-associated proteins during differentiation. In contrast with the saline-soluble fraction, no significant changes in Coomassie Blue-stained species of polypeptide could be detected during any phase of differentiation, that is during the time when major changes in transcription are occurring. Although no changes occur in the polypeptides stained by Coomassie Blue of the chromatin fraction, significant changes occur in the phosphorylated proteins, and these changes are totally different from those seen in the nuclear saline-soluble fraction. It is particularly notable that the greatest changes in the phosphorylation of chromatin-associated proteins occur at the two periods of greatest changes in gene expression, namely 0–2 h and 10–12 h. From these studies it is not possible to determine whether the changes in proteins and protein phosphorylation represent the cause or the effect of changes in gene transcription. However, the strong evidence pointing to phosphoproteins as effectors of gene transcription in other organisms leads one to consider it likely that the changes in the phosphorylation of these nuclear proteins represent early events, leading to changes in DNA transcription.

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