Phosphorylation of spore coat proteins during development of *Dictyostelium discoideum*

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Immunological evidence is presented which confirms that pp95, one of the major phosphoproteins accumulated in development of the cellular slime mould *Dictyostelium discoideum*, is identical with spore coat protein SP13. The site of phosphorylation is identified as phosphoserine. The second major phosphorylated component, pp74, corresponds to two co-migrating spore coat proteins known collectively as SP74.

After the depletion of nutrients, vegetative amoebae of *Dictyostelium discoideum* enter a developmental phase during which the cells aggregate via chemotaxis to form multicellular pseudoplasmodia. After a series of morphological changes, these eventually transform into fruiting bodies, each consisting of a group of spores supported on a cellular stalk. Cyclic AMP is the chemotactic agent involved and has also been implicated in the regulation of gene expression during development (Town & Gross, 1978; Williams *et al.*, 1980; Landfear & Lodish, 1980; Chung *et al.*, 1981). As a preliminary step in identifying the molecular events that may mediate this latter role, Coffman *et al.* (1981) examined the phosphorylation of proteins during *D. discoideum* development. Although several phosphoproteins were found to change in abundance early in development, after 14 h only two new phosphoproteins appeared and these had apparent mol.wts. of 74 000 (pp74) and 95 000 (pp95).

In 1979, Orlowski & Loomis characterized a group of polypeptides with mol.wts. 200 000 (SP200), 96 000 (SP96), 70 000 (SP70), 68 000 (SP68) and 60 000 (SP60) that were present in the coats of mature spores. SP96, SP70 and SP55 are accumulated late in development and one of them, SP96, is phosphorylated (Devine *et al.*, 1982). Therefore SP96 is probably the 'pp95' protein described by Coffman *et al.* (1981). Recently we have re-examined the spore coat proteins of *D. discoideum* and characterized components with mol.wts. of 170 000 (SP170), 103 000 (SP103),

97 000 (SP97), 90 000 (SP90), 82 000 (SP82), 76 000 (SP76) and 55 000 (SP55) (Wilkinson & Hames, 1983). Unlike the other spore coat proteins, SP103 is not disulphide-cross-linked in the spore coat and therefore corresponds to the SP96 protein described by Orlowski & Loomis (1979) that is similarly non-cross-linked. When analysed by one-dimensional SDS/polyacrylamide-gel electrophoresis, SP76 and SP72 often co-migrate to yield a single band with apparent mol.wt. 74 000 (SP74) (Wilkinson & Hames, 1983). Here we present evidence which confirms that SP103 is phosphorylated and identical with the major phosphoprotein pp95, and we identify the site of phosphorylation as phosphoserine. In addition we report that SP74 is also phosphorylated and presumably accounts for the other major phosphoprotein observed in late development, pp74 (Coffman *et al.*, 1981).

Materials and methods

Cell growth and development

Dictyostelium discoideum AX2 cells were grown and allowed to develop as described by Alton & Lodish (1977).

Radioisotopic labelling of cells

Cells at the 13 h stage of development were labelled with [³²P]phosphate by the labelling technique described by Wilkinson & Hames (1983), using 500 µCi of carrier-free [³²P]phosphate or 100 µCi of [³²S]methionine (both from Amersham International) per one-sixteenth of a Millipore filter.

Spore coat protein analysis

The protocols for sequential extraction of spores with SDS buffer and SDS/mercaptoethanol buffer, one-dimensional SDS/polyacrylamide-gel electrophoresis and fluorography have all been described

Abbreviation used: SDS, sodium dodecyl sulphate.

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elsewhere (Wilkinson & Hames, 1983). Immunoprecipitation of spore coat proteins was carried out by using an antibody raised by the injection of 1 mg of spore coat proteins, purified by the sequential extraction protocol (Wilkinson & Hames, 1983) and then emulsified with Freund's adjuvant, into male New Zealand White rabbits. After 4 weeks, a booster injection of 1 mg of spore coat proteins emulsified with Freund's incomplete adjuvant was administered and the rabbits bled after a further 1 and 2 weeks. After inactivation of complement by heating at 56°C for 30 min, immunoglobulin was prepared by two cycles of precipitation with $(\text{NH}_4)_2\text{SO}_4$ to 50% saturation and then exhaustively dialysed against 0.15 M-NaCl/10 mM-Tris/HCl, pH 7.5. Immunoprecipitation of spore proteins was carried out by using spore-protein antibody cross-absorbed with excess *D. discoideum* amoebal protein before use. For each portion of 5×10^5 developing cells (radiolabelled *in vivo*) to be immunoprecipitated, 50 μl (equivalent to 5×10^6 cells) of SDS/mercaptoethanol buffer extract of vegetative amoebae was mixed with 1 vol. of 10% (w/v) Triton X-100 and 8 vol. of 0.15 M-NaCl/0.05% NaN_3 /10 mM-Tris/HCl, pH 7.5. Aprotinin was also added to 1% final concentration. Next, 8 μl of spore-protein antibody was added and the mixture incubated at 22°C for 30 min for cross-absorption to occur. The SDS/mercaptoethanol extract of the radiolabelled developing cells was similarly diluted to 0.1% SDS/1% Triton X-100/1% Aprotinin, then the cross-absorbed antibody was added and the mixture incubated at 4°C for 60 min. Next, 4 mg (an excess) of Protein A-Sepharose (pre-swollen) was added and the mixture shaken gently at 4°C for 60 min. The Protein A-Sepharose with bound immunoglobulin was recovered by centrifugation at 14 000 g for 1 min, resuspended in immunobuffer (0.1% SDS/1% Triton X-100/0.15 M-NaCl/0.05 M- NaN_3 /10 mM-Tris/HCl, pH 7.5) and carefully layered on to 1 ml of 1 M-sucrose in immunobuffer in a 1.5 ml Eppendorf tube. After centrifugation at 17 000 g for 5 min the supernatant was removed and the pellet was washed twice by centrifugation (14 000 g, 1 min) each time with 1 ml of immunobuffer. The immunoprecipitated antigen was eluted from the Protein A-Sepharose by the addition of 50 μl of 2% SDS/5% β -mercaptoethanol/50 mM-Tris/HCl, pH 6.8, and heating at 100°C for 5 min. For SDS/polyacrylamide-gel-electrophoretic analysis, this whole mixture was then loaded into the sample well. After electrophoresis, all gels of ^{32}P -labelled proteins were equilibrated in 7% (w/v) trichloroacetic acid, then incubated with 7% trichloroacetic acid at 90°C for 30 min and finally washed gently, with shaking, with several changes of 7% trichloroacetic acid over a 4 h period. This procedure hydrolysed any nucleic acid contaminants

(especially when whole-cell extracts were analysed) without loss of the major phosphoproteins. After drying the gel, ^{32}P -labelled phosphoproteins were detected by indirect autoradiography (Laskey & Mills, 1977).

Analysis of the site of SP103 phosphorylation

Immunoprecipitated ^{32}P -labelled SP103 was resolved by SDS/polyacrylamide-gel electrophoresis and detected by indirect autoradiography. The gel segment corresponding to SP103 was excised and re-swollen in 0.05 M- NaHCO_3 /0.1% SDS containing 40 μg of bovine serum albumin/ml as protein carrier. The swollen gel was homogenized and then, after the addition of 2-mercaptoethanol to 5% (v/v) final concentration, the mixture was incubated at 100°C for 5 min and finally shaken at 22°C overnight. The eluate was recovered by centrifugation at 10 000 g for 10 min, the gel re-extracted as described above and the eluates pooled. Next, 37 μg of unlabelled spore coat protein, prepared by sequential extraction from mature spores (Wilkinson & Hames, 1983) was added as carrier and the solution was adjusted to 20% in trichloroacetic acid. After incubation at 0°C for 4 h, the protein precipitate was recovered by centrifugation at 10 000 g for 15 min, washed with ethanol and then ethanol/diethyl ether (1:1, v/v) and then air-dried overnight.

Acid hydrolysis of SP103 was performed in 6 M-HCl essentially as described by Hunter & Sefton (1980). After the removal of HCl by drying under reduced pressure, the hydrolysate was dissolved in a mixture of phosphotyrosine, phosphothreonine and phosphoserine (Sigma Chemical Co.), all at 1 mg/ml. The hydrolysate was analysed by electrophoresis on a 20 cm $\times$ 9 cm cellulose thin-layer plate (Eastman Kodak) in acetic acid/pyridine/water (10:1:189, v/v), pH 3.9, for 90 min at 500 V, with a sample of [^{32}P]phosphate co-electrophoresed in a separate lane as a marker. After electrophoresis, the phospho-amino acid markers were detected by staining with 1% ninhydrin/10% cadmium chloride (17:3, v/v). The plate was then covered with Clingfilm and examined by indirect autoradiography (Laskey & Mills, 1977) to detect ^{32}P -labelled phosphorylated amino acids and the [^{32}P]phosphate markers.

Results

The disulphide cross-linking of all the spore coat proteins in the spore coat except SP103 can be exploited for their rapid isolation using a sequential SDS buffer-SDS/mercaptoethanol buffer extraction protocol (Wilkinson & Hames, 1983). Intracellular spore proteins and most of SP103 were first extracted with SDS buffer (Fig. 1a) and then the disulphide-cross-linked spore coat proteins, plus residual SP103, were subsequently extracted with

Fig. 1. Phosphorylation of spore coat proteins SP103 and 'SP74'

Sample proteins were analysed by SDS/polyacrylamide-gel electrophoresis followed by fluorography (for ^{35}S) or indirect autoradiography (for ^{32}P). (a) and (b): proteins labelled by the addition of [^{35}S]methionine at 15 h of development and then extracted from mature spores (48 h) by SDS buffer (a) followed by SDS/mercaptoethanol buffer (b) respectively. (c) Those disulphide-cross-linked spore proteins (b) that were immunoprecipitated with spore-protein antibody. (d) Total cells proteins labelled with [^{32}P]phosphate from 13 to 48 h of development and extracted with SDS/mercaptoethanol buffer from mature spores. (e) Total proteins extracted directly with SDS/mercaptoethanol buffer from cells labelled with [^{35}S]methionine from 16 to 18 h of development followed by immunoprecipitation with spore-protein antibody. The band labelled SP97 + SP90 has been shown to consist of these two polypeptides by two-dimensional gel electrophoresis (Wilkinson & Hames, 1983). (f) Those ^{32}P -labelled phosphoproteins (lane d) which were immunoprecipitated with spore-protein antibody. (g-i) In a separate experiment, *D. discoideum* spores, labelled from 13 to 48 h of development with [^{32}P]phosphate, were sequentially extracted twice with SDS buffer at 100°C (lanes g and h) and then SDS/mercaptoethanol buffer at 100°C (i) followed by analysis of SDS/polyacrylamide-gel electrophoresis and fluorography. The molecular weights of the proteins shown were calculated from the distances migrated by a mixture of molecular-weight-marker polypeptides (Boehringer-Mannheim G.m.b.H.) co-electrophoresed on the same gels.

SDS/mercaptoethanol buffer (Fig. 1b). An antibody raised in rabbits by injection of the disulphide-cross-linked polypeptides immunoprecipitated all of the major spore coat proteins (Fig. 1c), including SP103, which was present in small amounts in the original antigen preparation. The immunoprecipitation was specific for the spore coat proteins under the experimental conditions used (Fig. 1e).

The profile of phosphoproteins accumulated late in development of *D. discoideum* was examined by addition of [^{32}P]phosphate at 13 h of development, followed by the extraction of mature fruiting bodies (at 48 h) with SDS/mercaptoethanol buffer. SDS/polyacrylamide-gel electrophoresis of the total cell extract followed by autoradiography showed several phosphoproteins, including two major components with apparent mol.wts. 103 000 and 74 000 (Fig.

1d). Immunoprecipitation with spore-protein antibody precipitated both these major phosphoproteins (Fig. 1f), indicating that these were the spore coat proteins SP103 and SP74 respectively. That the 103 000-mol.wt. phosphoprotein was indeed SP103 and not the closely migrating spore coat proteins SP97 or SP90 (Wilkinson & Hames, 1983) was shown by the fact that most of the 103 000-mol.wt. phosphoprotein was extracted in the absence of mercaptoethanol (Figs. 1g and 1h), a property unique to SP103 amongst the spore coat proteins (Orlowski & Loomis, 1979; Wilkinson & Hames, 1983; Figs. 1a and 1b). Phosphorylated SP74, as expected for disulphide-cross-linked spore coat protein, was extracted only in the presence of mercaptoethanol (Fig. 1i). The band corresponding to a phosphoprotein with a molecular weight of

approx. 200 000 (Figs. 1*d* and 1*f*) is probably phosphomyosin (Coffman *et al.*, 1981; Peltz *et al.*, 1981), some of which often co-purified together with the disulphide-cross-linked spore coat proteins, yet which can be clearly distinguished from SP170 by SDS/polyacrylamide-gel electrophoresis (Fig. 1*e*).

Quantitative densitometry of the corresponding autoradiographs indicates that SP103 is by far the

major phosphoprotein, accounting for approx. 94% of the spore coat protein phosphorylation observed (SP103 plus 'SP74') (D. G. Wilkinson & B. D. Hames, unpublished work).

The only site of phosphorylation of the major spore coat phosphoprotein, SP103, was phosphoserine, as analysed by acid hydrolysis of ^{32}P -labelled SP103 followed by thin-layer electrophoresis (Fig. 2).

Discussion

The immunological evidence presented here suggests that the two major phosphoproteins, pp95 and pp74, accumulated late in development of *D. discoideum* (Coffman *et al.*, 1981) are phosphorylated spore coat proteins SP103 and 'SP74'. The apparent discrepancy between the molecular weights of SP103 and pp95 is probably due to the anomalous behaviour of the spore coat proteins on SDS/polyacrylamide-gel electrophoresis (Wilkinson & Hames, 1983). Indeed, as described in the introduction, SP103 appears to be identical with SP96 identified by Orlowski & Loomis (1979). Spore coat proteins SP76 and SP72, which co-migrate as 'SP74' on SDS/polyacrylamide-gel electrophoresis, are synthesized at distinct times in development: SP76 first accumulates at 16–17 h, whereas SP72 arises only after culmination (D. G. Wilkinson & B. D. Hames, unpublished work). By comparison with the temporal pattern of pp74 accumulation (Coffman *et al.*, 1981), it is therefore likely that pp74 corresponds to SP76 rather than SP72.

Although the main site of SP103 phosphorylation is serine, the significant amount of phosphate released by acid hydrolysis may indicate the presence of more labile phosphate linkages, since phosphoserine is more than 90% stable under the hydrolysis conditions used (Bitte & Kabat, 1974). Phosphothreonine was not detected, even though it is less acid-labile than phosphoserine (Bitte & Kabat, 1974) and hence it is probably absent. Although phosphoserine usually occurs as a monoester (Wold, 1981), Gustafson & Milner (1980) have shown that essentially all of the phosphate residues in Proteinase I of *D. discoideum* occur in diester linkages, with each phosphate linked via phosphoester bonds both to a serine residue and also to an *N*-acetylglucosamine residue. Many of the spore coat proteins of *D. discoideum* are glycosylated (Orlowski & Loomis, 1979; Lam & Siu, 1981; Devine *et al.*, 1982; Wilkinson & Hames, 1983), including SP103, and hence phosphorylation of SP103 may also involve diester linkages of this kind.

Despite the wealth of knowledge concerning the phosphorylation of serine residues of key enzymes and its well-defined roles in metabolic regulation, the physiological role of structural-protein phos-

Fig. 2. *Determination of SP103 phosphorylation site* Samples were analysed by electrophoresis on a cellulose thin layer plate (see the Materials and methods section). (a), (b) and (d) contained phosphoserine (Ser-P), phosphothreonine (Thr-P) and phosphotyrosine (Tyr-P) standards respectively, and (c) contained a mixture of these phospho-amino acids. These were detected after electrophoresis by staining with ninhydrin. (e) and (f) contained an acid hydrolysate of SP103 and ^{32}P phosphate respectively, and the separated components were detected after electrophoresis by indirect autoradiography.

phorylation remains unclear. One possibility in the case of the spore coat proteins is that it allows ionic bridging by bivalent-metal cations and thereby supplements the disulphide cross-links that characterize the protein network within the mature spore coat of *D. discoideum*.

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