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Phosphoproteins in Dictyostelium discoideum

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The phosphoproteins of Dictyostelium discoideum were compared at different stages of development by polyacrylamide gel electrophoresis. Certain phosphoproteins of vegetative amoebae were conserved while others appeared and disappeared during development. Four major phosphoproteins with apparent subunit molecular weights of 50,000, 47,000, 38,000, and 34,000 disappeared precociously in response to exogenous cAMP. Two membranal phosphoproteins, with apparent subunit molecular weights of 80,000 and 81,000, appeared precociously in response to added cAMP. One of these phosphoproteins, molecular weight of 80,000, has been identified tentatively as the "contact site A" glycoprotein. Another membranal protein, with apparent subunit molecular weight of 42,000, unaffected in its appearance by cAMP, has been identified tentatively as phosphoactin.

Key words: membrane phosphoproteins, cAMP in development, Dictyostelium discoideum, phosphoproteins

The cellular slime mold, Dictyostelium discoideum, grows as a unicellular organism until its food supply is exhausted or the amoebae are transferred to an environment devoid of utilizable nutrients. Upon starvation, the amoebae begin a phase of morphogenesis and differentiation, resulting in a multicellular fruiting body with two specialized cell types, stalk cells and spore cells [1]. It has been well documented that in D discoideum cyclic AMP (cAMP) is required for the orderly assembly of the fruiting body. During aggregation cAMP acts as a chemotactic signal [2,3]. In addition, cAMP has been shown to affect the syntheses and activities of proteins throughout development [4–7].

As a preliminary step towards identifying the molecular events which might be responsible for the effects cAMP in D discoideum, we examined the influence of cAMP on the phosphorylation of proteins. We reasoned that cAMP may act by a mechanism involving phosphorylation catalyzed by a cAMP-dependent protein kinase. The occurrence of protein kinases in D discoideum has been reported [8,9]; the existence of a cAMP-dependent protein kinase has been claimed [8], but not confirmed [9–11]. We used sodium dodecyl sulfate-

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polyacrylamide gel electrophoresis (SDS-PAGE) to analyze the pattern of phosphoproteins formed during development and observed many changes. The changes occurred both during the formation of fruiting bodies on filters and during the acquisition of aggregation competence by amoebae in suspension. Exogenous cAMP altered the occurrence of certain phosphoproteins in amoebae starved in suspension.

Since phosphorylation of membranal proteins has been linked to changes in cellular behavior [12–14], we were especially interested in the possibility that phosphorylation of membranal proteins is crucial to the development of D discoideum. As a first step to this end, we examined the pattern of membranal proteins during development and the effect of cAMP on the temporal course of any changes. We observed, in fact, several changes in the pattern of membranal phosphoproteins during development. Of special interest were two membranal phosphoproteins (apparent subunit molecular weights 80,000 and 81,000) which appeared precociously in response to exogenous cAMP. The possibility that one of these phosphoproteins might be the "contact site A" glycoprotein is discussed. We also identified phosphoactin (pp42) as one of the membrane-associated phosphoproteins. Phosphoactin occurred at a relatively constant level in vegetative and developing amoebae independently of exogenous cAMP.

MATERIALS AND METHODS

Growth of Amoebae

D discoideum, strain Ax-3, clone RC-3, was obtained from Dr. David Soll, University of Iowa. Fresh cultures were started monthly from spores stored on silica gel [15] and were maintained at concentrations of between 10^4 and 6×10^6 amoebae per ml for 4–6 weeks. Doubling times in Medium HL-5 [16] supplemented with 50 mM glucose ranged from 10–12 hr at 22°C.

^{32}P-Labeling During Growth

Amoebae were grown in low phosphate medium MES-HL-5 [15] (per liter: 10 gm proteose peptone (Oxoid), 5 gm yeast extract (Difco), 10 gm glucose, 1.3 gm MES[2-(N-morpholino) ethane sulfonic acid, monohydrate], pH 6.6) to a concentration of either 1.5×10^6 (for log phase) or 9×10^6 amoebae/ml (for stationary phase). Then $^{32}P_i$ (carrier-free in 0.02 M HCl) was neutralized with 0.02 M NaOH and added to a concentration of 1 mCi $^{32}P_i/10^7$ amoebae. The amoebae were labeled for 8 hr.

^{32}P-Labeling During Development

To initiate differentiation by starvation, amoebae harvested at a density of $2–3 \times 10^6$/ml were washed by two centrifugations (3000 g) at 4°C in deionized, distilled H_2O.

Amoebae which were to develop on filters were resuspended at 10^8 amoebae/ml in MES-PDF [7] (per liter: 1.5 gm MES, 1.5 gm KCl, 0.6 gm $MgSO_4$, pH 6.5) containing 4 mCi/ml of neutralized $^{32}P_i$ (0.1 μM). The suspension was spread on purple membrane filters (Millipore Corp., average pore size 0.8 μm, diameter 4.7 cm). The filters were placed on cellulose absorbent sup-

port pads (Millipore Corp.) in 55-mm plastic dishes. The pads had been previously saturated with 1.5 ml of MES-PDF. There were 5×10^7 amoebae per filter and the concentration of $^{32}P_i$ was 2 mCi per filter. The amoebae were incubated at 22°C in a dark, humid chamber. Amoebae from two plates were sampled at each time point.*

Amoebae which were to develop in suspension were resuspended at 10^7 amoebae/ml in MES-LPS [15] (per liter: 1.5 gm MES, 1.5 gm KCl, 1.02 gm $MgCl_2 \cdot 6\ H_2O$, pH 6.0) containing 1 mCi/ml of neutralized $^{32}P_i$ (approximately 2×10^9 cpm/ml). The suspensions were incubated at 22°C with shaking at 190 rpm. Duplicate cultures of amoebae in suspension were exposed to pulses of either cAMP or 5′ AMP (control) every 7 min starting from the initiation of starvation such that the nucleotide concentration after each pulse was 10^{-7} M [6]. Two ml were sampled at each time point. The maximum uptake of $^{32}P_i$ by the amoebae was approximately 5% of the $^{32}P_i$ in the medium; 20% of the cellular $^{32}P_i$ was TCA-precipitable.

Preparation of Samples

Sampling was begun at 45 min after the initiation of starvation and subsequent samples were taken at 2-hour intervals for 24 hr. The samples were processed at 4°C except where specified. Growing amoebae, amoebae developing on filters, and amoebae suspended in buffer were treated in the following manner, respectively: 2×10^7 growing amoebae were harvested by sedimentation (3000 g) and resuspended in 3 ml of phosphate-buffered saline (PBS); 10^8 amoebae developing on filters were washed into 3 ml of PBS; and 2 ml of suspended amoebae were added to 1 ml of PBS.

The amoebae were washed by two centrifugations (300 g, 40 sec) in PBS. The washed pellets were resuspended in 2 ml of PBS and distributed for analysis of total phosphoproteins (0.5 ml) and membranal phosphoproteins (0.5 ml). The further processing of membranal fractions will be described below. The fraction used to analyze total phosphoproteins was prepared by a modification of the procedure of Garrels [17]. Samples were centrifuged (300 g, 40 sec), resuspended in 200 μl of 20 mM Tris-HCl, pH 8.8, 2 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), and quick-frozen in a dry ice bath to lyse the cells. The frozen sample was thawed in a 42°C water bath. Within 10 seconds, 24 μl of 3% (w/v) sodium dodecyl sulfate (SDS) containing 10% (v/v) β-mercaptoethanol (βME) were added and the sample vortexed. Within the next 10 sec, 24 μl of 3 mg/ml DNase I (Sigma) and 1.5 mg/ml RNase A (Sigma) in 0.5 M Tris-HCl, pH 7.0, 50 mM $CaCl_2$ were added and the sample again vortexed. Ten sec later 50 μl of 50% (w/v) glycerol, 25% (v/v) βME, 11.5% (w/v) SDS, 0.3 M Tris-HCl, pH 6.8, were added and the sample vortexed. A 20-μl aliquot was removed and frozen at –20°C (for precipitation with TCA, see below). The remainder of the sample was frozen in a dry ice bath and stored at –70°C (for analysis of phosphoproteins).

*Morphological development in MES-PDF buffer is normal during the first 16 hr. At later times development is retarded significantly, limited presumably by the lack of phosphate. Approximately 10% of the normal number of fruiting bodies appear after about 36 hr of starvation together with amoebal aggregates arrested at various post-16-hour stages of development.

Plasma membranes were isolated according to the method of Parish and Müller [18] with modifications as by Parish et al [19]. The washed amoebae were centrifuged at 300 g for 40 sec. The pellet was resuspended in 0.2 ml of PDF (per liter: 1.5 gm of KCl, 6.8 gm of KH_2PO_4, 0.5 gm of $MgCl_2 \cdot 6H_2O$, pH 6.5) containing concanavalin A, at a final concentration of 100 μg/ml, and incubated for 3 min at room temperature. Following the incubation, 1.0 ml of M Buffer (0.1 M Tris-HCl, pH 8.5, 0.1 M EDTA, 1 mM PMSF) was added and the preparation was centrifuged at 300 g for 40 sec. The pellet was washed by two centrifugations (300 g, 40 sec) in M Buffer. It was then resuspended in 0.2 ml of 0.2% (w/v) NP-40 (a gift from Particle Data Laboratories) in M Buffer and vortexed 2–3 sec. This procedure resulted in extensive disruption of the amoebae as viewed under the light microscope. The disrupted amoebae were diluted with 0.6 ml of M Buffer and centrifuged at 300 g for 10 sec to remove residual whole cells. The supernatant fluid was then centrifuged at 1700 g for 2 min and the membrane pellet was washed by two additional centrifugations (1700 g, 2 min) in M Buffer. It was suspended either in 100 μl of 2.3% SDS, 5% βME, 10% glycerol, 0.06 M Tris-HCl, pH 6.8, for one-dimensional analysis, or in 9.5 M urea, 2% (w/v) NP-40, 2% Ampholines (comprised of 1.6% pH 5–8 and 0.4% pH 3–10) and 5% βME for two-dimensional analysis. As judged by examination under the light microscope, this procedure completely solubilized the membranal fragments (no Coomassie-stainable or radioactive material was seen at the interface between the stacking gel and the resolving gels, or at the top of the stacking gel). A 20-μl aliquot was removed and frozen at −20°C (for precipitation with TCA; see below). The remainder of the sample was frozen in a dry ice bath and stored at −70°C (for analysis of phosphoproteins).

Plasma membranes were also isolated by the two-phase polymer procedure described for D discoideum by Siu et al [20]. Briefly, washed amoebae were swollen and fixed with $ZnCl_2$ and then disrupted in a Dounce homogenizer. The membranes were collected at the interface of a solution of Dextran 500 and polyethylene glycol and resuspended in the electrophoresis buffer mentioned above.

Eventually 0.2 ml of 10% (w/v) trichloroacetic acid (TCA) was added to the 20-μl aliquots, which were vortexed and centrifuged 5 min in an Eppendorf Centrifuge 3200. The supernatant fluid was removed by aspiration and the pellet was resuspended in 0.2 ml of 0.5 M NaOH. A 30-μl aliquot was mixed with 3.0 ml of liquid scintillation fluid and its radioactivity determined in order to estimate TCA precipitable material. The SDS which is present in the sample interferes with the TCA precipitation and reduces the radioactivity found in the TCA pellet by approximately 30%. Nonetheless, the radioactivity found in the TCA pellets served as an acceptable guide for normalizing the amount of non-TCA treated samples, such that TCA precipitable material corresponding to approximately 2×10^5 cpm was added to the individual lanes of the polyacrylamide gels. At this stage of our analysis, we disregarded changes in the specific activity of the [γ $-^{32}$P]-ATP pool under the different conditions since we were making comparisons of the *relative* band intensities on each lane.

Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gels were prepared according to the method of Laemmli [21]. The resolving gel contained 10% and the stacking gel 4.5%

acrylamide. All samples were quick thawed and boiled for 1 minute. Electrophoresis was performed at a constant voltage of 150V for 20–30 min (until the marker dye was at the interface between the stacking gel and the resolving gel), and then at 250V for approximately 2 hr. Gels were stained for 20 min in a solution of 0.1% (w/v) Coomassie Brilliant Blue R, 12.5% (w/v) TCA, 50% (v/v) methanol in H_2O, and then destained for 1–3 hrs in 10% (v/v) acetic acid, 6% (v/v) ethanol in H_2O. The gels were dried under vacuum onto Whatman 3MM paper and then placed in direct contact with Kodak X-omatic R film in a light-proof folder for 18–24 hr at −70°C.

The following proteins were used as molecular weight markers: phosphorylase b, 93,000; bovine serum albumin, 68,000; ovalbumin, 45,000; α-chymotrypsinogen, 25,000. The plot of log molecular weight versus centimeters of migration was linear (data not shown). Myosin (210,000) and β-galactosidase (130,000) were also employed as standards to estimate higher molecular weight proteins.

Two-Dimensional Gel Electrophoresis

Samples were analyzed by isoelectric focusing with 0.4% pH 3.5–10 and 1.6% pH 5–8 ampholines (LKB) followed by SDS-polyacrylamide gel electrophoresis [22]. The gel for the second dimension contained 10% acrylamide and 0.26% bisacrylamide. The pH profile was measured as described [22].

DNase I-Agarose Affinity Chromatography

DNase I-agarose was prepared by allowing 1 mg of DNase I to react with 1 gm of cyanogen bromide activated Sepharose 4B (Sigma) according to the method of Lazarides and Lindberg [23]. Unreacted sites were blocked with ethanolamine. A preparation of ^{32}P-labeled membranes (0.5 mg protein) was loaded onto the column. The column was washed and eluted as described [23].

RESULTS

We examined the phosphoproteins of vegetative amoebae harvested at two cell densities, 2×10^6/ml (log phase) and 1.3×10^7/ml (stationary phase), and of amoebae allowed to develop under two different conditions, on filters and in suspension. The pattern of phosphoproteins of developing amoebae did not change after 16 hr on either filters or in suspension and, consequently, gels of post-16-hour samples are not shown (see however, footnote on page 00). The Coomassie stainable proteins (not shown) and the ^{32}P-labeled material observed in the autoradiograms were sensitive to protease and resistant to DNase and RNase digestion.

Changes in Cellular Phosphoproteins

Phosphoproteins from ^{32}P-labeled lysates of vegetative and developing amoebae were compared after separation on an SDS-polyacrylamide slab gel and autoradiography. (Figs. 1–3). Table I is a summary of the major bands detected by autoradiography.

Vegetative amoebae. The phosphoprotein profiles from growing amoebae harvested at the two cell densities (Fig. 1) were similar, although minor differences were observed in some of the phosphorylated proteins (eg, phosphoprotein with subunit molecular weight of 45,000 [pp45]).

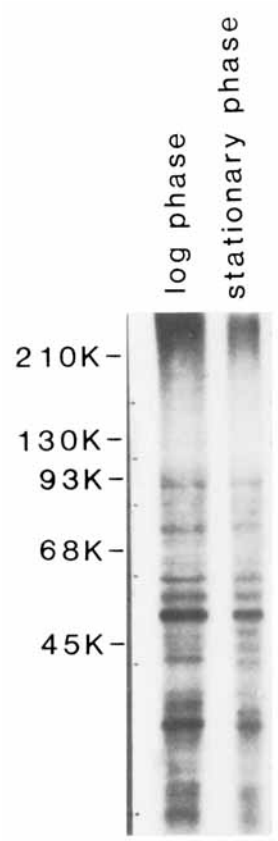

Fig. 1. Autoradiogram of a 10% SDS-polyacrylamide gel of ^{32}P-labeled lysates from log phase and stationary phase cultures of D discoideum. Standards for molecular weights were myosin (210,000), β-galactosidase (130,000), phosphorylase b (93,000), bovine serum albumin (68,000), ovalbumin (45,000), and α-chymotrypsinogen (25,000).

Amoebae developing on Millipore filters. There were many changes in the profile of phosphoproteins during development on Millipore filters (Fig. 2). It may be seen that phosphoproteins, additional to those already found in log phase vegetative amoebae, appeared after 45 min of development. (In a separate experiment, amoebae were labeled during the initial 45 min of starvation with a fivefold higher concentration of $^{32}P_i$; no additional changes in ^{32}P-phosphoproteins were observed.) Between 45 min and 2 hr of starvation several changes occurred. Thus, new bands appeared on the autoradiogram (eg, pp38); other bands increased in intensity (eg, pp50); while yet other bands decreased in intensity (eg, pp54). Between 2 hr and 6 hr of development there was little change in the pattern of phosphoproteins. After 6 hr, some of the bands in the autoradiogram started to disappear (eg, pp50, pp59, pp71). After 10 hr of development, two new bands appeared (pp74, pp95).

Amoebae in suspension: Effect of exogenous cAMP. Since cAMP profoundly affects the process of differentiation of D discoideum, we were especially interested to know whether cAMP altered the pattern of phosphoproteins

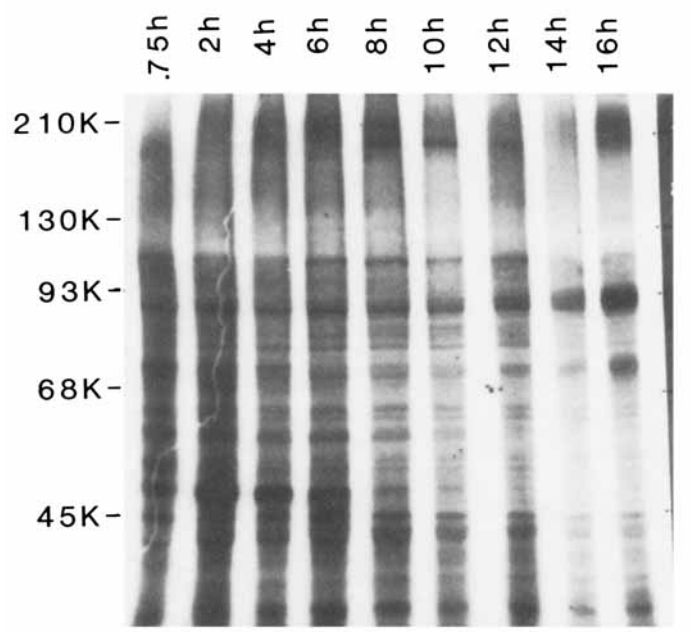

Fig 2 Autoradiogram of a 10% SDS-polyacrylamide gel of ^{32}P-labeled lysates from D discoideum during development on filters Standards as in Figure 1

during development. It proved inconvenient to study the effects of exogenous cAMP on the behavior of phosphoproteins in amoebae developing on filters. The inherent cAMP signaling and amplifying system is very effective and hence the influence of added cAMP is difficult to assess, particularly in view of the variable amounts of the cyclic nucleotide hydrolized by cAMP phosphodiesterases. Furthermore it is hard to distinguish between the effects of cAMP per se and those of cell-cell contact.

Therefore we allowed the amoebae to become aggregation-competent in shaken suspensions where cell to cell contact is minimal [6]. We exposed duplicate cultures of amoebae to pulses of cAMP or 5′ AMP (control) every 7 min such that the nucleotide concentration after every pulse was 10^{-7}M. It should be mentioned that the phosphoprotein profiles of extracts from cultures to which neither nucleotide had been added (data not shown) were identical with those of extracts from cultures to which 5′ AMP had been added (Fig. 3B). The autoradiograms corresponding to the phosphoproteins obtained from amoebae developing in suspension in the presence of cAMP (Fig. 3A) or 5′ AMP (Fig. 3B) showed several differences when compared to autoradiograms from amoebae developing on filters (Fig. 2). The most obvious example is pp38 which appeared as a major phosphoprotein during development in suspension, but was a minor component during development on filters. There were other quantitative differences (eg, pp36), and differences in the time course of appearance and disappearance of some phosphoproteins (eg, pp50) in amoebae which developed under the two different conditions.

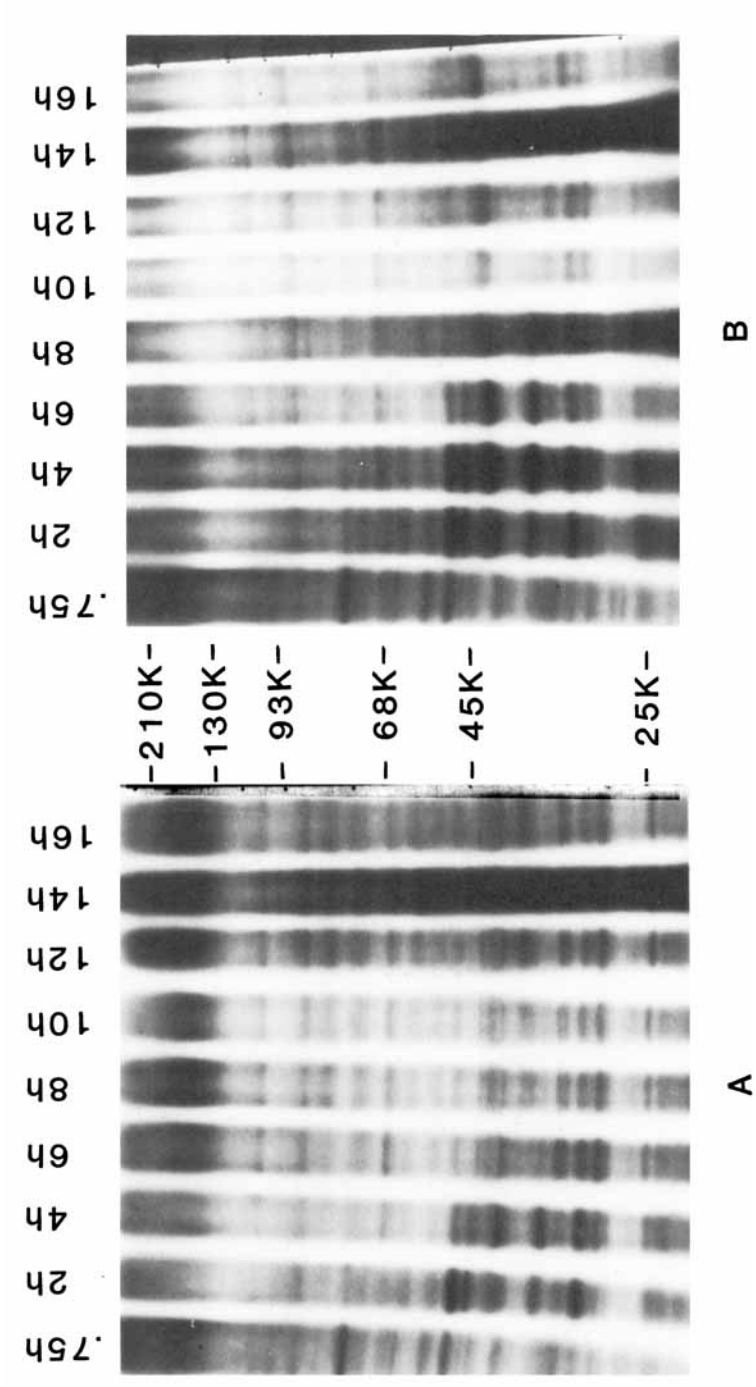

Fig. 3 Autoradiograms of 10% SDS-polyacrylamide gels of ^{32}P-labeled lysates from suspensions of starving D discoideum to which cAMP was added (A); or to which 5' AMP was added (B). Aliquots from 4-hour samples were found to be sensitive to protease digestion. Standards as in Figure 1

TABLE I. Phosphoproteins of Dictyostelium discoideum

Mol wt (10^{-3})	Vegetative amoebae: Log phase	Stationary phase	Filters[a]: 0.75 hr	2 hr	4 hr	6 hr	8 hr	10 hr	12 hr	14 hr	16 hr
95	—[c]	—	—	—	—	—	—	—	—	+	++
74	±	±	±	±	±	±	±	±	±	+	++
71	+	+	+	+	+	+	+	+	±	—	—
64	+	+	+	+	+	+	+	+	+	±	±
59	+	+	+	+	+	+	+	±	±	±	±
54	+	+	+	±	±	±	±	±	±	±	±
50	++	++	++	+++	++	++	+	±	±	±	±
47	+	+	±	+	+	±	—	—	—	—	—
38	—	—	—	±	±	±	±	±	±	±	±
36	+	+	±	±	±	±	±	±	±	±	±
34	+	+	+	+	+	+	+	+	+	±	±

Mol wt (10^{-3})	Developing amoebae, Suspension: 5′A cA[b] 0.75 hr		5′A cA 2 hr		5′A cA 4 hr		5′A cA 6 hr		5′A cA 8 hr		5′A cA 10 hr		5′A cA 12 hr		5′A cA 14 hr		5′A cA 16 hr	
95	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
74	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
71	+	+	±	±	±	—	±	—	±	—	—	—	—	—	—	—	—	—
64	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
59	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
54	+	+	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
50	+	+	++	++	++	+	+	±	±	±	±	±	±	±	±	±	±	±
47	+	+	++	++	++	+	+	—	—	—	—	—	—	—	—	—	—	—
38	—	—	++	++	+++	++	++	—	+	—	—	—	—	—	—	—	—	—
36	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
34	+	+	++	++	++	++	++	+	+	+	+	+	+	+	+	+	+	+

[a]Morphology observed: 6 hours (ripple), 8 hours (loose aggregates), 10 hours (tight aggregates), 12 hours (finger), 14 hours (early culmination), 16 hours (maxi-finger) [40].
[b]5′ A = 5′ AMP; cA = cAMP.
[c]The symbols indicate relative intensities: —, band absent; ±, faint band; +, clear band; ++, heavy band.
Framed symbols indicate significant changes in the phosphoprotein profile associated with the addition of cAMP to amoebae in suspension.

The addition of cAMP caused the premature disappearance of four major phosphoproteins (pp34, pp38, pp47, pp50), although the appearance of these four phosphoproteins was independent of cAMP. Thus pp38, pp47, and pp50 began to disappear at 4 hr in the presence of cAMP and at 6 hr in the control, while pp34 began to disappear at 6 hr in the presence of cAMP and at 8 hr in the control.

Changes in Membranal Phosphoproteins During Development

Membranal phosphoproteins from vegetative and developing amoebae isolated by the concanavalin A-Triton procedure were compared after separation on an SDS-polyacrylamide slab gel and autoradiography (Figs. 4–6). Table II is a summary of the major bands detected by autoradiography. The phosphoproteins of membranes isolated by an alternate method, the aqueous two-

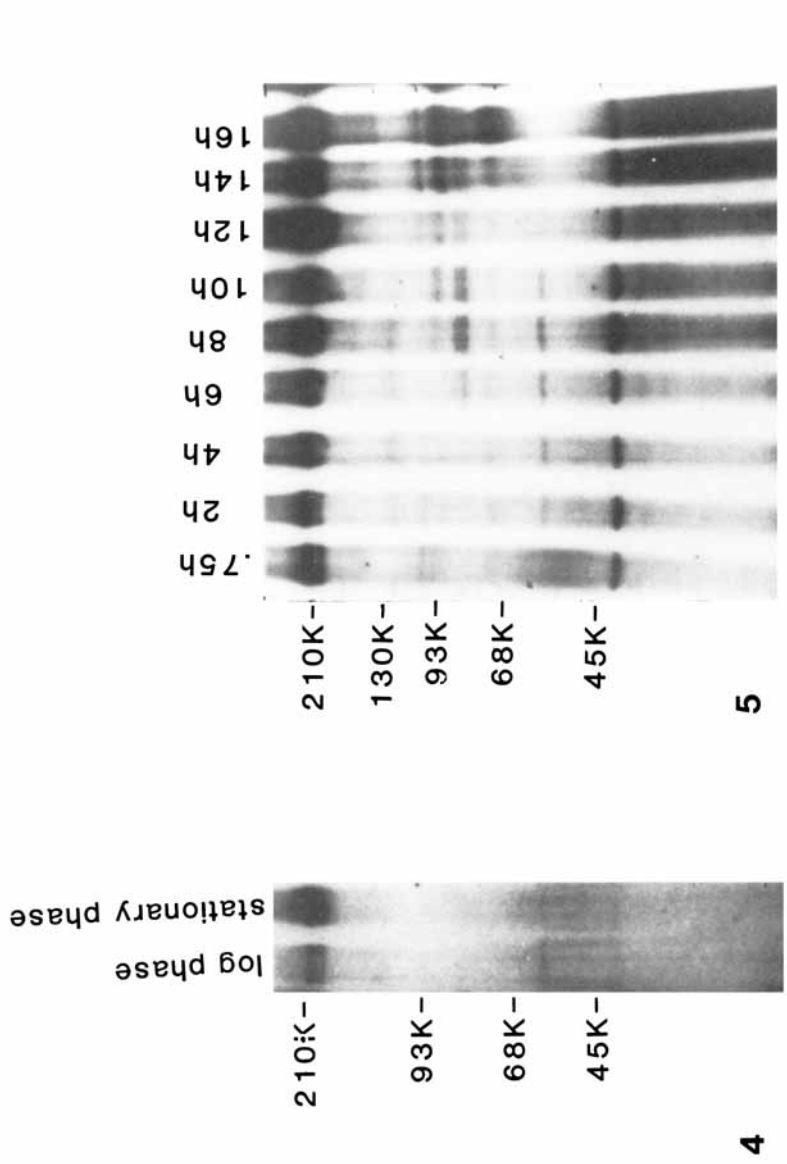

Fig 4 Autoradiogram of a 10% SDS polyacrylamide gel of ^{32}P-labeled membranal proteins from log phase and stationary phase cultures of D discoideum Standards as in Figure 1

Fig 5 Autoradiogram of a 10% SDS-polyacrylamide gel of ^{32}P-labeled membranal proteins from D discoideum during development on filters Aliquots from the 8-hour sample were found to be sensitive to protease digestion Standards as in Figure 1

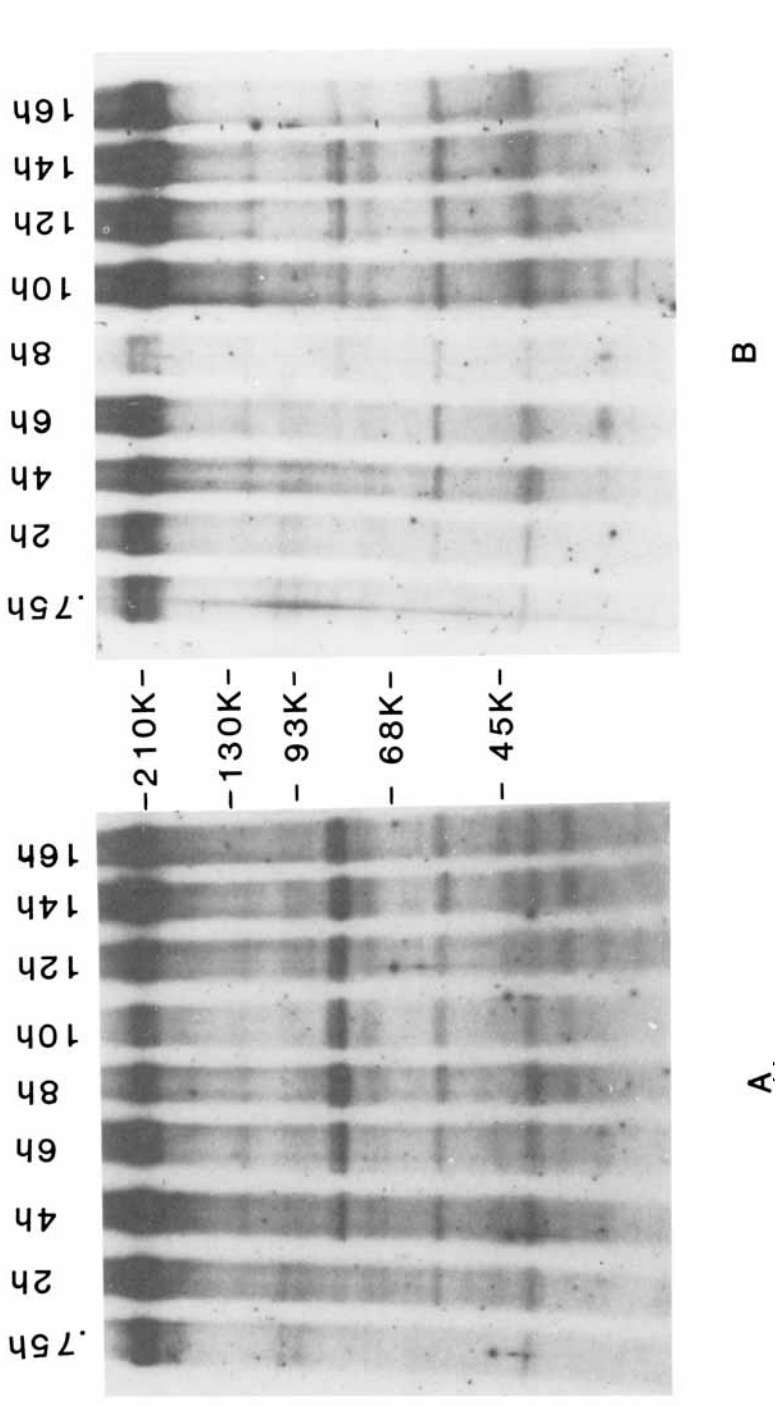

Fig 6 Autoradiograms of 10% SDS-polyacrylamide gels of ^{32}P labeled membranal proteins from suspensions of starving D discoideum to which cAMP was added (A), or to which 5 AMP was added (B) Standards as in Figure 1

TABLE II. Membranal Phosphoproteins of Dictyostelium discoideum

Mol. wt (10^{-3})	Vegetative amoebae: Log phase	Vegetative amoebae: Stationary phase	Filters[a]: 0.75 hr	2 hr	4 hr	6 hr	8 hr	10 hr	12 hr	14 hr	16 hr
210	+[c]	+	+	+	+	+	+	+	++	++	++
95	±	±	±	±	±	±	±	+	+	++	++
81	—	—	—	—	—	±	+	+	+	+	+
80	—	—	—	—	—	+	+	+	+	+	+
74	±	±	±	±	±	±	±	±	±	+	++
64	+	+	±	+	+	+	+	+	+	±	±
42	+	+	+	+	+	+	+	+	+	+	+

Developing amoebae

Mol wt (10^{-3})	Suspension: 5'A cA[b] 0.75 hr		5'A cA 2 hr		5'A cA 4 hr		5'A cA 6 hr		5'A cA 8 hr		5'A cA 10 hr		5'A cA 12 hr		5'A cA 14 hr		5'A cA 16 hr	
210	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
95	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
81	—	—	—	—	—	±	—	+	—	+	±	+	+	+	+	+	+	+
80	—	—	—	—	—	+	—	+	—	+	+	+	+	+	+	+	+	+
74	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
64	±	±	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
42	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a]Morphology observed: 6 hours (ripple), 8 hours (loose aggregates), 10 hours (tight aggregates), 12 hours (finger), 14 hours (early culmination), 16 hours (maxi-finger) [40].
[b]5'A = 5'AMP; cA = cAMP.
[c]The symbols indicate relative intensities: —, band absent; ±, faint band; +, clear band; ++, heavy band.
Framed symbols indicate significant changes in the phosphoprotein profile associated with the addition of cAMP to amoebae in suspension.

phase polymer procedure described by Siu et al [20], produced a similar phosphoprotein pattern (data not shown).

The pattern of membranal phosphoproteins of vegetative amoebae (Fig. 4) was identical with that of amoebae which had developed on filters (Fig. 5) or in suspension (Fig. 6) for the first 45 minutes. However, successive stages of development were reflected in the changing phosphoprotein profile. A phosphoprotein of apparent molecular weight 64,000 (pp64) was present at low levels in growing amoebae and increased in concentration between 45 min and 2 hr of development, both in amoebae starved on filters and in amoebae starved in suspension. Both pp74 and pp95 increased in abundance late in development on filters but remained at a low level throughout starvation in suspension. During development on filters, pp80 and pp81 appeared at 6 hr. In suspension, in the absence of exogenous cAMP (Fig. 4B), pp80 and pp81 were retarded in their

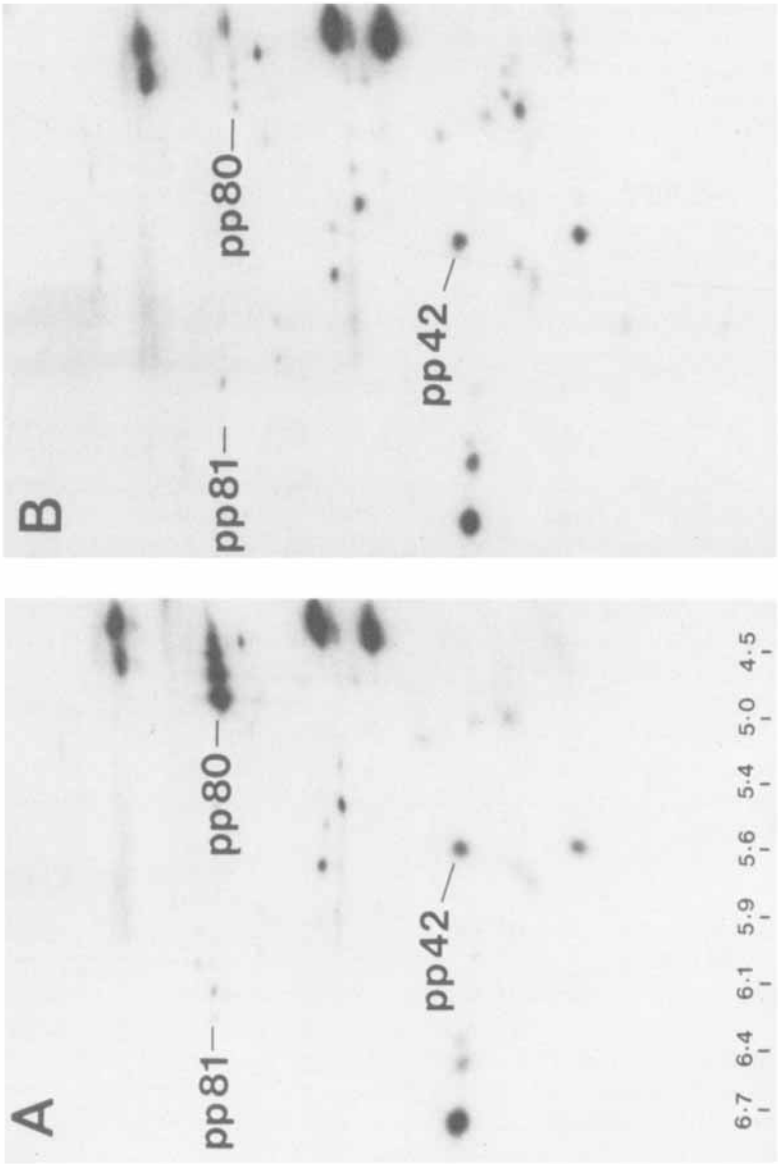

Fig. 7. Autoradiograms of portions of two-dimensional gels of ^{32}P-labeled membranal proteins from suspensions of *amoebae at 4 hours of development in the presence of* cAMP (A) or 5' AMP (B). The positions of pp42, pp80 (multiple spots), and pp81 (multiple spots) are designated. An approximate calibration of the pH gradient of the isoelectric focusing gel is shown on the horizontal axis of A.

appearance (10 hr), while the addition of cAMP (Fig. 4A) caused their precocious appearance (4 hr). In an attempt to identify pp80, we partially purified a ^{32}P-labeled membrane fraction from amoebae which had developed for 4 hours in the presence of exogenous cAMP. We employed butanol extraction at pH 5.5 in a procedure which Müller et al [24] used for the purification of the "contact site A" glycoprotein. The method yielded an approximately 30-fold enrichment of pp80 in the aqueous phase (data not shown).

We were interested to know whether the bands on the autoradiogram from the one-dimensional gels represented a composite of phosphoproteins which might be resolved using high resolution two-dimensional gel electrophoresis. Amoebae starving in suspension were labeled in vivo with ^{32}P-orthophosphate as described above. Autoradiograms of portions of the two-dimensional gels from membranes prepared after 4 hr of development in the presence of either cAMP (Fig. 7A) or 5′ AMP (Fig. 7B) are presented. We conclude that the bands from the one-dimensional gels represent, for the most part, individual phosphoproteins (except pp42). In particular, pp80 and pp81 which are altered in the time course of their appearance by cAMP, as seen in the one-dimensional analysis (Fig. 6), represent in fact only two phosphoproteins. Furthermore, the isoelectric point of pp80 (pH 4.5) differs distinctly from that of pp81 (pH 6.1) and these are, therefore, two different proteins rather than one protein varying in its state of modification. Inspection of the autoradiograms indicates that both pp80 and pp81 have multiple forms which are phosphorylated.

The spot labeled pp42 on the two-dimensional autoradiogram (Fig. 7) occupies a position very close to, and on the acidic side of, that corresponding to actin. Actin binds strongly to DNase I [23]. When ^{32}P-labeled membranes from developing amoebae were examined by DNase I-agarose affinity chromatography, the observed counts in the 3 M guanidine-HCl eluate (3 M guanidine-HCl quantitatively and specifically elutes bound actin [23]), were equivalent to the counts predicted, on the basis of one-dimensional and two-dimensional gel electrophoresis, to be in phosphoactin. Furthermore, the fraction which eluted in 3M guanidine-HCl was found to have a subunit molecular weight of 42K on SDS-PAGE (data not shown).

DISCUSSION

The phosphoproteins of lysates of developing amoebae change drastically during development. We have grouped the major phosphoproteins of amoebal lysates into two classes on the basis of their appearance and disappearance during development. There is a class of phosphoproteins (pp26, pp28, pp29, pp32, pp45, pp54, pp61, pp79, pp81, pp90, pp110, pp125) which were found in vegetative as well as in starving amoebae at all stages of growth and development at approximately constant concentrations. It is unlikely that these phosphoproteins play a crucial role in development.

A second class of phosphoproteins either appeared or disappeared at various times during development (cf Table I). While there was not an exact correspondence between the changes in phosphoproteins in amoebae developing in suspension and on filters, we note that all the changes which occurred in amoebae on filters prior to 6–10 hr were also found in amoebae starved in suspension (eg, pp47, pp50). There were a few changes in amoebae in suspension

(pp34, pp38 at late time points) which did not occur in amoebae on filters. Changes which occurred after 10 hr of development on filters (eg, pp74, pp95) did not occur in cells in suspension and may require cell-cell contact. A subset of this second class are those phosphoproteins which were unaffected in their appearance by exogenous cAMP, but whose disappearance was brought about prematurely by exogenous cAMP (pp34, pp38, pp47, pp50). Other proteins, eg, discoidin, also disappear prematurely in response to cAMP [25].

A two-dimensional analysis of the phosphoproteins in the lysate would, no doubt, reveal additional phosphoproteins, some of which might appear precociously in response to cAMP. Furthermore, analysis of discrete cellular fractions may well reveal additional phosphoproteins and suggest their function on the basis of location. Therefore, we determined the distribution of phosphoproteins in the plasma membrane. There are several methods for the preparation of membranes from D discoideum [18,26–30]. We used the concanavalin A-Triton technique described by Parish and Müller [18] for two reasons. From a conparison of the procedures for the preparation of membranes, we concluded that the concanavalin A-Triton method yields membranes relatively free of contaminating cells, organelles, or soluble proteins. In addition, the concanavalin A-Triton method is rapid and minimizes the effects of proteases and phosphatases. To control for possible artifacts due to concanavalin A stabilization of the membrane or to detergent lysis, we also isolated membranes from amoebae at selected time points by the aqueous two-phase polymer procedure described for D discoideum by Siu et al [20]. This procedure produced a similar, possibly identical phosphoprotein pattern (data not shown). Additional evidence that the phosphoproteins which co-fractionated with membranes were membranal phosphoproteins rather, than impurities which are nonspecifically absorbed, comes from analysis of the phosphoprotein profiles. The major phosphoproteins in the total lysate were not detected in the purified membranes (compare Figs. 2 and 5). The more prominent membranal phosphoproteins (eg, pp42, pp74, pp95) were minor components of the phosphoprotein profile in the total lysate. Indeed, visualization of changes in the minor membranal components (eg, pp80, pp81) required the enrichment for these proteins which is gained by fractionation of the membranes.

One membranal phosphoprotein (pp42) occurred at a relatively constant level in vegetative and developing amoebae. On two-dimensional gels (Fig. 7) pp42 migrated to a position very close to, but on the acidic side of, a protein which we assumed to be actin based on its isoelectric point, ie, extrapolated to be pH 5.6 from Fig. 1A in [7]. The association of actin with membranes in D. discoideum has been previously reported [31,32]. By the criterion of binding to DNase I, an affinity reagent for actin [23], pp42 is a phosphorylated form of actin. Phosphorylated actin has been found in the S49 mouse lymphoma cells [33] and in Chinese hamster ovary cells [34]. The physiological significance of the co-fractionation of the presumptive phosphoactin with plasma membranes is not clear; although this association was observed in membranes isolated by several different procedures (see also [31]), a contamination of the membranes by cytoplasmic actin or phosphoactin, respectively, is not ruled out. By the same token the occurrence of pp210, presumably phosphomyosin [35,36], in the membranal fraction has to be interpreted with caution.

Two membranal phosphoproteins (pp74, pp95) increased in abundance late in development on filters (after 10 hr), but remained at a low level during starvation in suspension. Possibly the formation of pp74 and pp95 requires cell-cell contact which does not occur when amoebae are starved in suspension. One of these phosphoproteins, pp95, corresponds to a glycoprotein, apparent molecular weight 95,000, described by Steinemann and Parish [37] with respect to apparent molecular weight and temporal pattern of accumulation.

We suggest that pp80 may be a phosphorylated form of the glycoprotein of "contact site A" (csA), based on several analogous properties of pp80 and csA. The time of appearance of pp80 under several conditions (filters, suspension with cAMP pulses, and suspension without cAMP pulses) is similar to the time of appearance of csA as reported in the literature [38,39]. The apparent molecular weight of pp80 is identical with the molecular weight of csA [24]. Butanol extraction of membranes by the method used by Müller et al [24] in the purification of csA results in an approximately 30-fold enrichment for pp80 in the aqueous base [Coffman DS and Rickenberg HV, manuscript in preparation]. The isoelectric point of pp80 (pH 4.5) is very similar to that of csA [Dr. Ben Murray, personal communication]. Experiments are in progress to confirm the identity of pp80 and csA.

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