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A PROTEIN KINASE OF THE PLASMA MEMBRANE OF *DICTYOSTELIUM DISCOIDEUM*

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D. discoideum **amoebae were found to phosphorylate plasma membrane proteins when intact cells were incubated with either [γ-^{32}P]ATP or [^{32}P]phosphate. In the first case, the incorporation was largely a consequence of the hydrolysis of [γ-^{32}P]ATP, cellular uptake of the generated [^{32}P]phosphate and its subsequent incorporation into ATP. When the contribution of this process to the phosphorylating activity of intact cells was eliminated, an ecto-protein kinase (ATP: protein phosphotransferase, EC 2.7.1.37) activity could be demonstrated. As amoebae progressed through their aggregation program, they showed a decreased ability to phosphorylate their plasma membrane when incubated with [γ-^{32}P]ATP or [^{32}P]phosphate. Analysis of ATPase activity, permeability properties and the pattern of proteins phosphorylated by intact cells and isolated plasma membranes lead to the following conclusions: the lower levels of phosphorylation observed with starved cells reflected an altered uptake of [^{32}P]phosphate by these cells rather than a significant change in the plasma membrane protein kinase activity. Neither the substrates nor the activity of the ecto-protein kinase was dramatically altered during starvation.**

Introduction

For the slime mold amoebae, *D. discoideum*, external cyclic AMP is the primary messenger for cell-cell communication. The information for chemotaxis and cell differentiation is encoded in the form of extracellular cyclic AMP pulses [1–4]. The effects of cyclic AMP are believed to be mediated by a plasma membrane cyclic AMP receptor(s) [5]. The number of these receptors increases as cells undertake their aggregation program and demonstrate enhanced sensitivity to external cyclic AMP. Several laboratories have reported that binding of cyclic AMP to the cell surface is periodic, although there is disagreement concerning the frequency of these oscillations (2 or 5 min) and their biological relevance [6,7]. The molecular events subsequent to cyclic AMP binding to cells are unclear but do involve a rapid (within 15–30 s) activation of adenylate cyclase [8]. Activation and deactivation of this enzyme is also rhythmic and occurs with a periodicity of 5 min [7,9]. The rapidity with which these two events occur in *D. discoideum* amoebae suggest that they reflect modifications of the components in question and not changes in their synthesis and degradation. In light of the known mechanism of cyclic AMP action in other systems [10], we have examined the possible involvement of plasma membrane phosphorylation in the processing of the chemotactic signal by slime mold amoebae. Previously published information concerning the cell surface phosphorylation and the presence of an ecto-protein kinase in this system is contradictory. Intact cells have been reported to incorporate [γ-^{32}P]ATP into acid precipitable material [6,11–13]and in one case [6], this activity has been shown to oscillate with the same frequency as binding to the cyclic AMP receptor. Reports from other laboratories indicate that intact cells do not phosphorylate plasma membrane components when incubated with [γ-^{32}P]ATP [14–16]. The aims of

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Abbreviations: Mes, 4-morpholineethanesulfonic acid; PMSF, phenylmethylsulphonyl fluoride.

the experiments reported here are to determine if (1) plasma membrane proteins are phosphorylated; (2) there is a plasma membrane protein kinase or ecto-protein kinase; (3) this activity exhibits an oscillatory behavior and (4) either the protein kinase or its protein substrates are affected by cyclic AMP.

Experimental procedures

Strains and culture conditions. Ax-2 amoebae [17] were maintained as exponentially growing cultures ($1-2 \cdot 10^6$ cells/ml) in HL-5 medium [18]. Cells were starved as spinner suspensions [19] in 20 mM Mes buffer pH 6.4 at a density of 10^7 cells/ml buffer. Viability of the cells under different culture conditions was determined by cloning 1500 amoebae on SM plates as described by Sussman [18]. Plating efficiency was determined using an exponentially growing culture and found to be greater than 95%.

Phosphorylation of intact cells. In a final volume of 0.1 ml, $2 \cdot 10^6$ cells were incubated at 21°C in 20 mM Mes buffer, pH 6.4, $5 \cdot 10^{-4}$ M EGTA and either $2 \cdot 10^{-6}$ M [γ-^{32}P]ATP or [^{32}P]phosphate (10–20 Ci/mmol), Amersham). The reaction was terminated by the addition of nonradioactive ATP or sodium phosphate to a final concentration of 10^{-3} M. If both uptake and acid precipitable material were being examined, cells were then washed twice with 20 mM phosphate buffer pH 6.4. Aliquots were either counted immediately for measurements of total cellular radioactivity or after precipitation with 10% TCA on 3 MM filters. When samples were analyzed by SDS-polyacrylamide gel electrophoresis, the reaction was terminated by the addition of sample buffer (25 mM Na_2CO_3/25 mM dithiothreitol/1% SDS/10% sucrose) and immediately boiled for 2 min. All experiments were performed in duplicate and repeated a minimum of three times.

Phosphorylation by isolated plasma membranes. Plasma membranes were isolated on polylysine-coated acrylamide beads as described by Cohen et al. [20] and modified for *D. discoideum* by Jacobson [21]. In some cases, 1 mM PMSF was added to all the buffers. As with other cells [20,22,23] such preparations are enriched approx. 10–12-fold in plasma membrane markers and essentially devoid of other subcellular marker enzymes [24]. Plasma membranes attached to beads were phosphorylated in the same incubation mixture as intact cells. When the effects of cyclic nucleotides were examined, 10 mM dithiothreitol was sometimes added to inhibit the plasma membrane phosphodiesterase. The reaction was arrested by washing the beads twice with 20 mM Mes pH 6.4/1 mM ATP/5 mM sodium phosphate/2 mM sodium pyrophosphate. The beads were then immediately resuspended in sample buffer, vortexed, boiled for 2 min and again vortexed for 2 min. Beads were removed by centrifugation and the supernatant used for SDS-polyacrylamide gel electrophoresis. Where indicated, plasma membranes were extracted from the beads prior to phosphorylation. This was performed by vigorously vortexing the beads in high salt (0.5 M K_2HPO_4) as described by Cohen et al. [20]. Plasma membranes were reisolated by diluting the high salt supernatant into 10 mM Tris-HCl buffer pH 8 and centrifuging at $105\,000 \times g$ for 45 min. Membranes were washed by resuspending them in 10 mM Tris-HCl buffer pH 8 and reisolated by centrifugation.

SDS-polyacrylamide gel electrophoresis. Slab-gel electrophoresis was carried out as described by Chua and Bennoun [25] using a polyacrylamide gradient of 7.5–15%. Gels were stained with Coomassie blue and molecular weights were determined from a standard curve using proteins of molecular weights between 21 000 and 98 000 (Bio-rad). For autoradiography, gels were exposed to Kodak RP1 film at −70°C in Dupont cassettes containing lightning plus intensifying screens.

Thin layer chromatography. Hydrolysis of added [γ-^{32}P]ATP by intact cells was followed by incubating amoebae as described above for various times, removing them by centrifugation, and chromatographing aliquots of the supernatant on either PEI-cellulose developed in 1.5 M LiCl or cellulose thin layer plates developed in butanol/acetic acid/H_2O (5 : 2 : 3). Nonradioactive ATP was added to each sample prior to chromatography to allow for its localization under ultraviolet irradiation. The appropriate spots were cut out and counted in a scintillation counter. Synthesis of ATP was assessed by incubating cells with [^{32}P]phosphate for various times, after which aliquots were precipitated in 10% TCA at 4°C. Samples were centrifuged at $7000 \times g$ for 5 min and then analyzed by two-dimensional chromatography according to Cashel et al. [26].

Radioactive areas were identified by autoradiography after a 14 h exposure.

Partial characterization of the phosphorylated products. Cells or plasma membranes were incubated with either [γ-^{32}P]ATP or [^{32}P]phosphate. After different times, aliquots were precipitated with 10% TCA. Precipitates were either maintained at 0°C, heated in 10% TCA at 80°C for 10 min, heated in 1 N NaOH at 100°C for 15 min, or extracted with chloroform/methanol (2 : 1, v/v), chloroform/methanol/H_2O (1 : 1 : 3, v/v), or submitted to hydroxylamine treatment according to Hokins et al. [27]. All samples were washed extensively with 10% TCA, extracted with ethanol/ether and than either counted or analyzed by SDS-polyacrylamide gel electrophoresis.

Results

Incorporation of [γ-^{32}P]ATP into trichloroacetic acid-precipitable material by intact cells

As seen in Fig. 1A, cells harvested from exponentially growing cultures incorporated [γ-^{32}P]ATP into trichloroacetic acid-precipitable material at an approximately linear rate during a 20 min incubation period. This incorporation was proportional to the number of cells added (from 5 to 20 · 10^6 cells/ml)

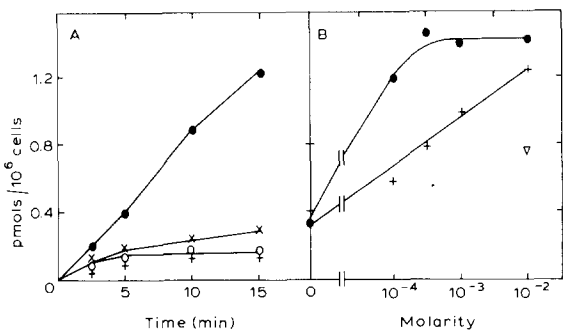

Fig. 1. Characteristics of [γ-^{32}P]ATP incorporation by intact cells. (A) Vegetative (•———•); 2 h-starved (X———X); 4 h-starved (○———○) and 6 h-starved (+———+) amoebae were incubated with [γ-^{32}P]ATP. At the indicated times, aliquots were trichloroacetic acid-precipitated on 3 MM filters. (B) Vegetative amoebae were incubated with [γ-^{32}P]-ATP in the presence of various concentrations of $MnCl_2$ (•———•); $MgCl_2$ (+———+) or $CaCl_2$ (△). Aliquots were trichloroacetic acid-precipitated on 3 MM filters after a 10 min incubation.

as well as the temperature at which the assay was performed. At 4°C incorporation decreased by 90% as compared to that observed at 21°C. At neither temperature did the presence of either 10^{-6} or 10^{-3} M cyclic AMP alter the rate or extent of acid-insoluble material formed. When cells were placed under starvation conditions to initiate their developmental program, a rapid and extensive decrease in the radioactivity incorporated was observed. The majority of the phosphorylated products were formed during the first 5 min of incubation with a slight, gradual increase occurring later. Neither an inhibitor nor activator of the phosphorylation reaction was detected when 6-h starved and vegetative cells were mixed and their levels of phosphorylation measured. It has been reported that amoebae incorporate [γ-^{32}P]ATP into trichloroacetic acid-precipitable material with a periodicity of 2 min [6]. In experiments so designed, we were unable to detect any oscillations in [γ-^{32}P]ATP incorporation into acid-insoluble material using either vegetative or starved amoebae.

The ionic preferences for the incorporation of radioactivity into trichloroacetic acid-precipitable material are depicted in Fig. 1B. This experiment employed vegetative cells but similar results were obtained using aggregation competent cells. Optimum incorporation of [γ-^{32}P]ATP occurred with a Mn^{2+} concentration of 5 · 10^{-4} M. Mg^{2+} could substitute for Mn^{2+} but higher concentrations were required to obtain maximum activity. Less incorporation was observed when Ca^{2+} was present as the sole source of divalent cation. Low levels of acid-precipitable radioactivity were obtained in the absence of added ions. Approx. 56–66% of the radioactivity remained as ATP after a 10 min incubation with either vegetative or aggregation competent amoebae. The same result was obtained when the Mn^{2+} concentration was raised to 5 · 10^{-3} M or when $MnCl_2$ was replaced by $MgCl_2$. Thus, the lower incorporation of radioactivity into trichloroacetic acid-insoluble material seen with starved cells or when Mg^{2+} was present cannot be attributed to differences in ATPase activity.

The nature of the labeled acid-insoluble material was examined as described earlier. Approx. 90% of the radioactivity precipitated by acid was released upon treatment of the precipitate with 1 N NaOH. The majority of the radioactivity was resistant to

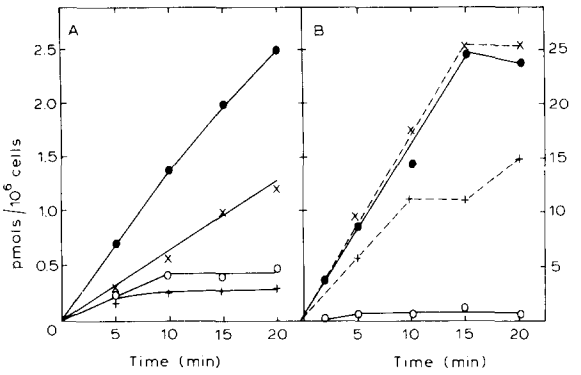

Fig. 2. Characteristics of [^{32}P]phosphate incorporation by intact cells. (A) Vegetative (●———●); 2 h-starved (X———X); 4 h-starved (○———○) and 6 h-starved (+———+) amoebae were incubated with [^{32}P]phosphate. At the indicated times, aliquots were trichloroacetic acid-precipitated on 3 MM filters. (B) Vegetative amoebae were incubated with [^{32}P]phosphate (●———●; X------X) or [^{32}P]phosphate plus 10^{-3} M sodium azide (○———○; +------+) and the amount total (X------X; +------+) and trichloroacetic acid-insoluble (●———●; ○———○) radioactivity determined. The levels of trichloroacetic acid-soluble material are indicated on the right-hand ordinate.

both hydrolysis by hot trichloroacetic acid and extraction with chloroform/methanol or ethanol/ether. No radioactivity was released by hydroxylamine treatment. These properties suggest that the majority of the radioactive-labeled components are proteins phosphorylated via their serine or threonine residues.

No trichloroacetic acid-insoluble material was formed when [γ-^{32}P]ATP was substituted with either [^{3}H]ATP or [α-^{32}P]ATP. The incorporation of radioactivity into trichloroacetic acid-precipitable material by intact cells apparently does not reflect the uptake of ATP by cells. The possibility that amoebae took up the radioactive phosphate generated by ATP hydrolysis and subsequently used it to phosphorylate cellular components were also examined. We observed that when amoebae were incubated with [^{32}P]phosphate of the same specific activity as our [γ-^{32}P]ATP, a large amount of radioactivity entered the cells and became acid precipitable (Fig. 2A). Formation of trichloroacetic acid-insoluble material paralleled the rate of uptake of the [^{32}P]phosphate by the cells. Starved cells took up less radioactivity and correspondingly less was found as trichloroacetic acid-insoluble material. Partial characterization of the phosphorylated material indicated them to be proteins probably phosphorylated via their serine or threonine residues. Unlike when cells were incubated with [γ-^{32}P]ATP, incorporation of [^{32}P]phosphate into acid insoluble material was the same when cells were incubated with either Mg^{2+} or Mn^{2+}. Uptake of [^{32}P]phosphate was also not affected by the nature of the ion present. Incorporation was dependent upon the ability of cells to synthesize ATP: when vegetative cells were incubated with 10^{-3} M sodium azide for 15 min and the [^{32}P]phosphate added, formation of radioactive trichloroacetic acid-precipitable material was extensively decreased (Fig. 2B). By 10 min, cells incorporated less than 10% of the radioactivity of untreated cells. Although azide-treated cells took up less radioactivity, this could not account for the drop in trichloroacetic acid-precipitable material. Analysis of [^{32}P]phosphate incorporation into ATP indicated a 70% decrease occurred within 15 min of incubation with sodium azide. The degree to which incorporation of [γ-^{32}P]ATP into acid-precipitable material reflects the metabolism of the [^{32}P]phosphate generated by ATP hydrolysis was investigated in the following experiments.

Profile of phosphorylated products and their localization on plasma membranes

The proteins labeled with [γ-^{32}P]ATP by cells which had been starved for different periods were identified by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 3). Independent of their state of starvation, cells incubated with ATP for 1 min incorporated radioactivity into 7–8 bands (see lane 1 for representative example). A difference in labeling between vegetative and starved cells was observed only when longer incubation periods were used to radioactively-label proteins (lanes 2–9). The complex pattern seen with vegetative cells incubated for 10 min with [γ-^{32}P]ATP became progressively simplified as cell starvation time increased. By 5.5 h of cell starvation (lane 6), the majority of the label was found in 7–8 proteins, the same as those phosphorylated when labeling was performed during a 1 min incubation (lane 1). In some experiments, cells were starved for either 30 min or until they expressed aggregation competence. Aliquots of cells were removed from the population every minute and

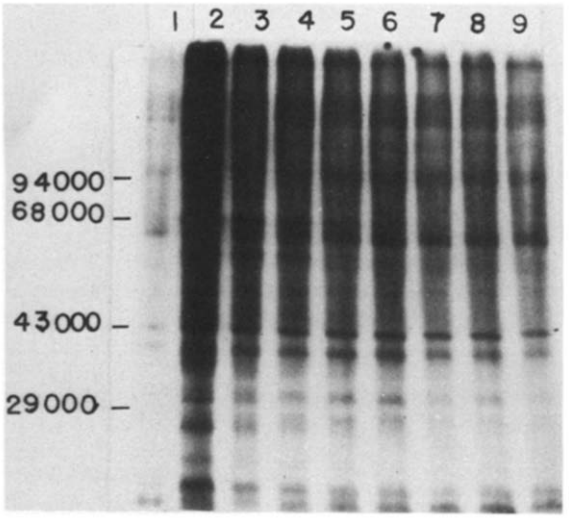

Fig. 3. Autoradiogram of proteins phosphorylated during development. Vegetative amoebae (lanes 1 and 2) or amoebae which had been starved for 1 h (lane 3); 2 h (lane 4); 3.5 h (lane 5); 5.5 h (lane 6); 7 h (lane 7); 8.5 h (lane 8) and 10 h (lane 9) were incubated with [γ-^{32}P]ATP for 1 min (lane 1) or 10 min (2–9).

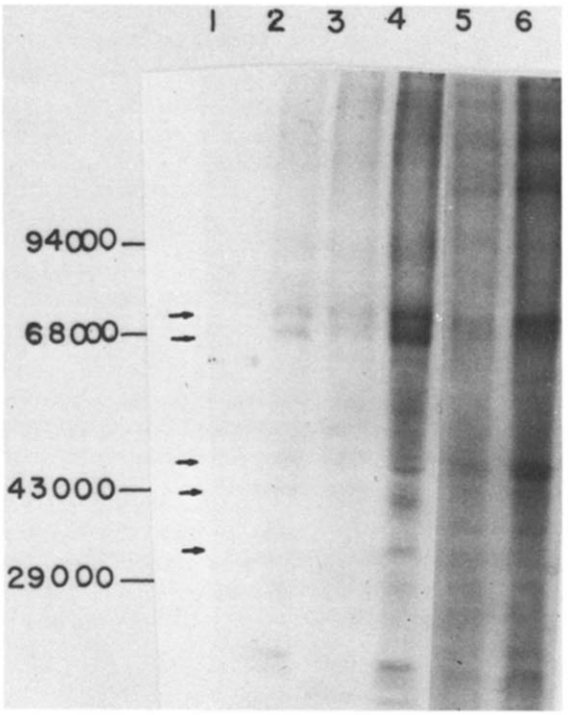

Fig. 4. Autoradiogram of the proteins phosphorylated using [γ-^{32}P]ATP or [^{32}P]phosphate. Vegetative amoebae were incubated for 1 min (lanes 1, 2) or 5 min (lanes 3–6); with [^{32}P]phosphate (odd numbered lanes) or [γ-^{32}P]ATP (even numbered lanes). Lanes 1–4 show the profile of labeled proteins from intact cells; 5 and 6 that of the subsequently isolated plasma membranes.

incubated with [γ-^{32}P]ATP for either 30 s or 1 min. SDS-polyacrylamide gel electrophoresis analysis indicated no changes in the labeling profile during the 20 min period of the experiments.

The proteins phosphorylated when vegetative amoebae were incubated with either [γ-^{32}P]ATP or [^{32}P]phosphate were compared (Fig. 4). During a 1-min incubation, little radioactivity was detected when cells were incubated with [^{32}P]phosphate in contrast to the significant labeling of 7–8 bands with [γ-^{32}P]ATP (lanes 1 and 2). By 5 min, the pattern observed with phosphate-treated cells was, for the most part, the same as that obtained with cells incubated with [γ-^{32}P]ATP. Some preferential labeling of the bands indicated by the arrows did occur with [γ-^{32}P]ATP (lanes 3 and 4). Longer reaction times did not reveal any new bands. In these experiments, plasma membranes were isolated from cells which had been incubated with radioactive phosphate or ATP for increasing time periods (lanes 5 and 6). Some differences in the labeling pattern of isolated plasma membranes and intact cells were noticed. However, if the phosphorylated proteins of plasma membrane preparations were compared to those of cells which had been treated as though their plasme membranes were to be isolated, no differences were found. Thus, our washing and incubation procedures required for the preparation of plasma membranes resulted in some change in labeling profile compared to cells which had not undergone these treatments. Since these changes were not associated with a release of phosphorylated substances into the medium (data not shown), they probably reflect a protein phosphatase activity. Labeled membranes extracted from polylysine-coated beads by high salt contained the same radioactive-labeled proteins as membranes which were solubilized directly from the beads. These findings confirm the fact that the majority of the phosphorylated proteins detected on intact cells are membrane bound and also indicate that they are integral plasma membrane components.

A preferential utilization of ATP to label plasma membrane proteins was also evidenced by preincuba-

ting cells with sodium azide and/or nonradioactive phosphate for 10 min. After such treatment, vegetative amoebae were incubated for 1, 2 or 5 min with either [γ-^{32}P]ATP, [γ-^{32}P]ATP + 10^{-4} M sodium phosphate, [γ-^{32}P]ATP + 10^{-4} M sodium phosphate + 10^{-3} M sodium azide. SDS-polyacrylamide gel electrophoresis showed that the pattern of proteins phosphorylated during the first min was unchanged by the presence of azide and/or phosphate. With longer incubation times (Fig. 5), a number of phosphorylated proteins were not labeled by cells incubated with phosphate and/or azide (lanes 1–3). The proteins which continued to be labeled under these various conditions were similar to those phosphorylated when untreated cells were incubated with [γ-^{32}P]ATP for 1 min. Different results were obtained when [^{32}P]phosphate was used to label cells (lanes 4–6). The addition of nonradioactive phosphate to the incubation mixture did not inhibit the radioactive-labeling of selected bands but instead caused a general diminution of labeling (lane 5). At all times, the presence of azide totally inhibited the phosphorylation of intact cells with [^{32}P]-phosphate (lane 6). Similar experiments were performed using cells which had been starved for 6–7 h. Proteins were radioactive-labeled only when [γ-^{32}P]-ATP was used as a phosphate donor and the profile of labeled bands was unchanged by the addition of nonradioactive phosphate and azide. The proteins identified under these conditions were similar to those observed when vegetative amoebae were incubated with [γ-^{32}P]ATP in the presence of azide. Apparently there is a plasma membrane protein kinase which can utilize external ATP to phosphorylate plasma membrane proteins.

Critical in the interpretation of these data is that this ecto-protein kinase activity is not an artifact due to cell damage or lysis and release of intracellular protein kinases. This aspect was explored in the following experiments. Vegetative or aggregation competent amoebae were incubated for either 1 or 10 min with our phosphorylating mixture minus any radioactive chemical. Amoebae were removed by centrifugation and the supernatants incubated with [γ-^{32}P]ATP. In some cases histone or casein was added as an exogenous substrate for any protein kinase which might have been released. No phosphorylated compounds were detected either by tri-

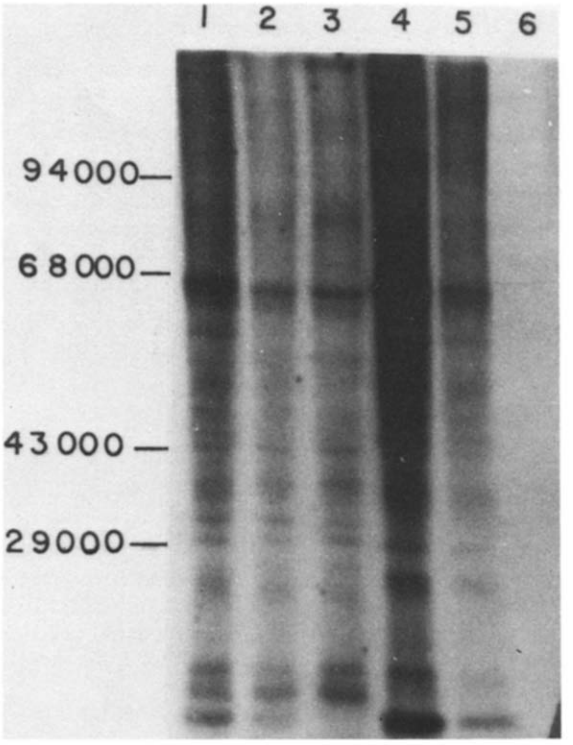

Fig. 5. Proteins phosphorylated by azide-treated cells using [γ-^{32}P]ATP and [^{32}P]phosphate. Vegetative amoebae were incubated for 10 min with 10^{-4} M sodium phosphate (lanes 2 and 5) or phosphate plus 10^{-3} M sodium azide (lanes 3 and 6). [γ-^{32}P]ATP (lanes 1–3) or [^{32}P]phosphate (4–6) was added. After 5 min the reaction was terminated and the samples analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

chloroacetic acid precipitation or SDS-polyacrylamide gel electrophoresis. Since the components phosphorylated by intact cells were found to be integral membrane proteins, the inability to detect a protein kinase or phosphorylated proteins in the medium was not due to their adsorption to the cells. When the pattern of proteins phosphorylated by broken cell preparations was examined, it was found to be significantly different from that observed using intact cells. These results would argue against the possibility that the ecto-protein kinase activity reflects the activity of damaged cells. Further evidence was obtained by examining the viability of the cell population. This was monitored by cloning cells (see Experimental procedures) which had been incubated with our phosphorylating mixture for various time periods up to 30 min. When the number of clones arising were compared to those obtained when

exponentially growing cells were similarly plated, no cell damage was observed. These findings were consistent with other experiments using trypan blue staining as an indication of cell viability.

Isolated plasma membranes retain protein kinase activity

Plasma membranes isolated on polylysine-coated beads were incubated with [γ-^{32}P]ATP and the phosphorylated products analyzed by SDS-polyacrylamide gel electrophoresis. It should be pointed out that these experiments require not only that the protein kinase maintain its activity but that the acceptor proteins also be present. Membranes from vegetative or starved amoebae labeled approx. 14–16 bands within the first minute of incubation with [γ-^{32}P]ATP (see Fig. 6, lane 1 for results using starved cells). When Mn^{2+} was replaced by the same concentration of Mg^{2+} or Ca^{2+}, little radioactivity was incorporated. The presence of 10^{-2} M NaF in the incubation mixture resulted in a greater degree of labeling, suggesting a plasma membrane phosphatase was also present. The kinase in question preferentially uses ATP as the phosphate donor since the addition of GTP did not alter the pattern or degree of phosphorylation. The presence of 10^{-6} or 10^{-3} M cyclic AMP or cyclic GMP had no demonstrable effect. Susceptibility to the different hydrolytic conditions indicated that the phosphorylated compounds were proteins. When phosphorylated membranes were extracted from beads by high salt and analyzed by SDS-polyacrylamide gel electrophoresis, bands similar to those seen using membranes which had not been extracted from the beads were observed (lane 2). Thus, labeling was confined to integral membrane proteins and not significantly contaminated by cytoplasmic components. If membranes were first released from the beads and then incubated with [γ-^{32}P]ATP, they incorporated radioactivity into similar proteins as membranes which were still attached to beads (lane 3).

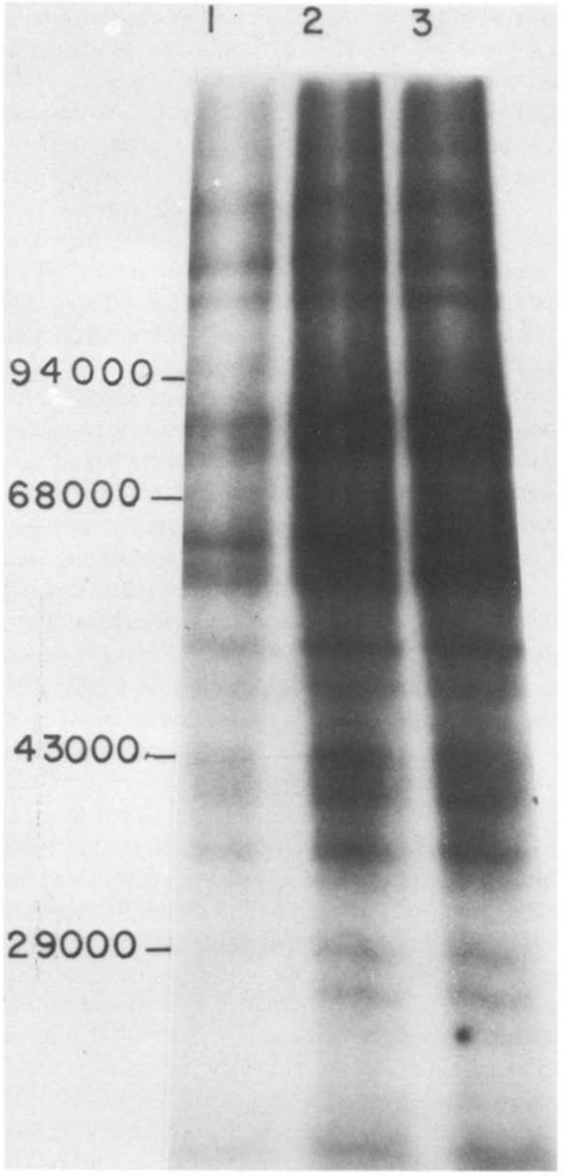

Fig. 6. Protein kinase activity of aggregation-competent cells. Plasma membranes were prepared from 6 h-starved cells and incubated with [γ-^{32}P]ATP for 10 min (lanes 1 and 2). Membranes were then extracted from the beads with high salt (lane 2). Membranes were first extracted and then incubated for 10 with [γ-^{32}P]ATP (lane 3). Similar findings were observed using plasma membranes from vegetative cells.

Discussion

The data presented in this communication provide evidence for a protein kinase(s) located in the plasma membrane of *D. discoideum* amoebae which phosphorylates plasma membrane proteins. This phosphorylating activity, monitored on plasma membranes isolated from either vegetative or aggregation competent cells did not appear to be affected by cyclic AMP or cyclic GMP. However, since some of

our results suggest that plasma membranes may contain a protein phosphatase, it may be premature to classify the protein kinase described here as cyclic AMP independent. It is possible that some regulation by the cyclic nucleotide will materialize when the activity of the phosphatase is better understood.

Phosphorylation of the cell surface was not only demonstrated using isolated plasma membrane fragments, but also by incubating intact cells with [γ-^{32}P]ATP or [^{32}P]phosphate. Although the same plasma membrane proteins were labeled when cells were incubated with either radioactive chemical, [γ-^{32}P]ATP was initially a more efficient phosphate donor: Within 1 min, 7–8 plasma membrane proteins were phosphorylated when cells were incubated with [γ-^{32}P]ATP. Little labeling was observed within this time period when [^{32}P]phosphate was used. With longer incubation times, these proteins were preferentially labeled when cells were incubated with [γ-^{32}P]ATP as opposed to [^{32}P]phosphate. Phosphorylation of those proteins by [γ-^{32}P]ATP was not eliminated when endogenous ATP synthesis was inhibited. Under such conditions no proteins were phosphorylated by [^{32}P]phosphate. Since no cell damage or lysis was detectable, these data would suggest that cells can use exogenous ATP directly to phosphorylate plasma membrane proteins. It was not possible to obtain further evidence for this ecto-protein kinase activity by monitoring the phosphorylation by intact cells of exogenous substrates since the addition of histone or casein to the medium causes cells to become leaky or otherwise release a soluble protein kinase (Ref. 21 and unpublished data).

When intact starved cells were incubated with [γ-^{32}P]ATP, less radioactivity was incorporated into trichloroacetic acid-precipitable material compared to vegetative cells and only 7–8 proteins were labeled. ATP hydrolysis occurred to the same extent with vegetative or starved cells but the latter were found to take up little [^{32}P]phosphate. Since the protein kinase of isolated membranes from starved cells phosphorylated a larger number of plasma membrane proteins, it appears that the total potential of the kinase activity of starved cells could not be realized due to the decreased labeling of the intracellular ATP pool. What was realized was the ecto-protein kinase activity: during a 10-min incubation, starved cells could use only [γ-^{32}P]ATP to phosphorylate proteins and that this activity was unaltered when endogenous ATP synthesis was inhibited by azide. The proteins phosphorylated were similar to those labeled by vegetative cells incubated with [γ-^{32}P]ATP and azide. It is not clear if the ecto-protein kinase activity described here is a separate enzyme or if the plasma membrane protein kinase, capable of utilizing intracellular ATP to phosphorylate plasma membrane components, is responsible. It has recently been reported that *D. discoideum* amoebae release ATP into the medium at concentrations which can attain 10^{-6} M [28]. Thus, if a distinct ecto-protein kinase is responsible for the phosphorylating activity of intact cells, then the substrate for this enzyme could be available.

King and Frazier [6] have reported 2 min oscillations in phosphorylation as measured by trichloroacetic acid precipitation when cells were incubated with [γ-^{32}P]ATP. We were unable to detect such changes when phosphorylation was examined not only by acid precipitation but by SDS-polyacrylamide gel electrophoresis as well. Since no information concerning either the stability of the radioactive ligand, or the biochemical nature or subcellular localization of the material phosphorylated in the experiments of King and Frazier is available, it is difficult to assess the reasons for our differences. Some of the possible artifacts of the technique used by King and Frazier have been discussed by Rahmsdorf et al. [16].

Acknowledgements

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