

Endogenous Cyclic AMP-Dependent Phosphorylation
in vitro
of Cytosolic Proteins from
Dictyostelium discoideum

by

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INTRODUCTION

The Problem of Development

Development is an orderly and progressive sequence of changes, resulting in a steady increase in complexity (Torrey, 1971). A fertilized egg develops into a complex human being; a bud develops into a flower; an ant society develops into a prescribed social pattern. Since the term is used to describe a number of distinct, but often overlapping events, a general classification scheme might include the following processes: growth, morphogenesis, and differentiation. This research may have bearing on present understanding of these processes, so it will be useful now to define them.

Growth implies a developmental increase in mass. It results from synthesis of new cytoplasmic and nucleic protoplasm, and usually results in cell division and/or cell enlargement. Morphogenesis is the generation of new form, a term which is most commonly used to designate the formative movements of multiplying cells. Morphogenetic movements are those movements of cells or protoplasm, as distinct from cell division. The increasing diversification of form and function which occurs in an organelle, cell, or tissue structure is referred to as differentiation. It is by this

process that cells become different from one another and also different from what they were originally. At the root of this diversification are the myriad molecular changes that determine the course of development: biochemical differentiation.

During development of even the simplest eukaryotic organisms, two or three of these processes may occur simultaneously. The developmental biologist is not only interested in understanding the mechanisms which underly these processes, but also the nature of the various control mechanisms which determine the amount of growth, the consistency of morphogenetic movements, and the proportionment of differing cell types within individuals and species.

Bonner(1974) suggests a straightforward way to bring together the concepts of growth, morphogenetic movements, and differentiation to address the problem of their collective control. Recalling that reproduction occurs in successive reproductive cycles, and that "particular substances appear at certain times in specified places", it is important to find out (1) the nature of these substances and how they are produced, (2) when they are produced, and (3) where they are placed.

Biochemists and molecular geneticists have provided much of present-day understanding of the first problem. Very simply, the ultimate source of the developmental program resides in the genome. Along with certain enzymes

which assist in the process, DNA makes an RNA template from which proteins are synthesized in the cytoplasm. The proteins, some of which are enzymes, then determine the cell's eventual functional role in the organism.

The problem of synthetic control is encompassed in Bonner's second topic, the timing mechanisms. Most of the questions that are addressed in my research also come under this category. Though prokaryotes have been studied more than eukaryotes, in both cases synthetic control is considered to be ultimately at the level of transcription, translation, and enzyme activity. Eukaryotic cells are more complex in that they contain a great deal of information in the form of previously-made DNA products such as plastids, mitochondria, and Golgi bodies. With each new cell division, then, certain informational molecules are "inherited" from the cytoplasm and there are many more possible points of control.

The information that is necessary for a developmental program appears in the form of control-loops which may be entirely cytoplasmic or involve nuclear-cytoplasmic interaction. Also to be considered are the substances called inducers and hormones which in multicellular eukaryotic organisms traverse from one group of cells to another and determine the course of biochemical differentiation. This topic will be discussed in greater detail later (see A Pro-

posed Role for Cyclic AMP in Development). Though many stage-specific biochemical processes are known to exist (reviewed by Loomis, 1975), there is only rudimentary understanding of the control mechanisms that function during development. The question still remains: What are the mechanisms which determine the alternate fates an undifferentiated cell may take?

A generally-accepted model envisions differentiation as a successive cascade of biochemical steps. A small change in one substance (an initial substrate, for example) may completely alter the final state of the cell, the result being two stable cell types which differ in function and/or appearance. These steady states may be set up in a number of ways: by small changes in cytoplasmic enzymes, nuclear activities, inductors, hormones, local pH, temperature, or humidity. One can cite several examples in plants where alternate differentiation pathways may be traced to such defined morphogenetic events. Skoog and Miller (1957), for instance, found that tobacco callus tissue would produce root tissue, leaf and bud tissue, whole plant shoots, or callus, depending on the relative concentrations of auxin and cytokinin with which they were treated. Even in this case, where cause and effect seem almost tangible, a mechanism of action for the differentiation process itself is unknown.

The last category of inquiry involves the localization of substances within developing organisms. Bonner(1974) lists several possibilities which may account for the placement of substances in a consistent pattern. These include molecular bonding, cell adhesion, diffusion, polar and active transport, electrophoresis, cell movements, and growth movements. The exact importance of each of these processes in development will necessarily await a further understanding of pattern-formation.

Ultimately, it is the goal of the developmental biologist to understand how these cellular substances are able to effect a cell's correct orientation in space. The process is no doubt complex, and little could be gained from studying higher organisms. Instead, the fundamental principles are best explored in simple multicellular organisms. The cellular slime mold, Dictyostelium discoideum, is a popular model system of eukaryotic development, for reasons outlined in the next section. Though the limitations of any model system should not be ignored, a basic understanding of a biochemical process in one organism can usually shed light on related processes in other systems.

Dictyostelium as a Model System for Development

Dictyostelium discoideum is a cellular slime mold, typically found in nature as a single-celled amoebae feeding on bacteria and other matter in the forest soil (Loomis, 1975). Under favorable conditions, they reproduce by binary fission. When deprived of a food source, however, the cells begin their developmental program leading to formation of a fruiting body (Fig. 1). The amoebae first aggregate in response to the chemotactic agent cyclic AMP (Konijn et al., 1968), and then collect in large streaming patterns to form groups of up to 100,000 cells (Loomis, 1975). A surface sheath is deposited and the aggregate migrates horizontally as a pseudoplasmodium or slug. At the slug stage, the anterior third of the slug is composed of presumptive stalk cells; the posterior two-thirds is composed of presumptive spore cells (Brenner, 1977; Bonner, 1971; Farnsworth and Loomis, 1974; Gregg and Badman, 1970). After a variable amount of time, the cell mass rights itself at the culmination stage, and the anterior tip rises vertically to begin formation of the fruiting body. The anterior cells differentiate into stalk cells, first by entering into the top of their secreted casing and moving downward. Then the cells swell by becoming filled with a vacuole, and eventually die, their rigid cellulose walls remaining as a slender supporting stalk (Raper and Fennel, 1952). Posterior cells in the

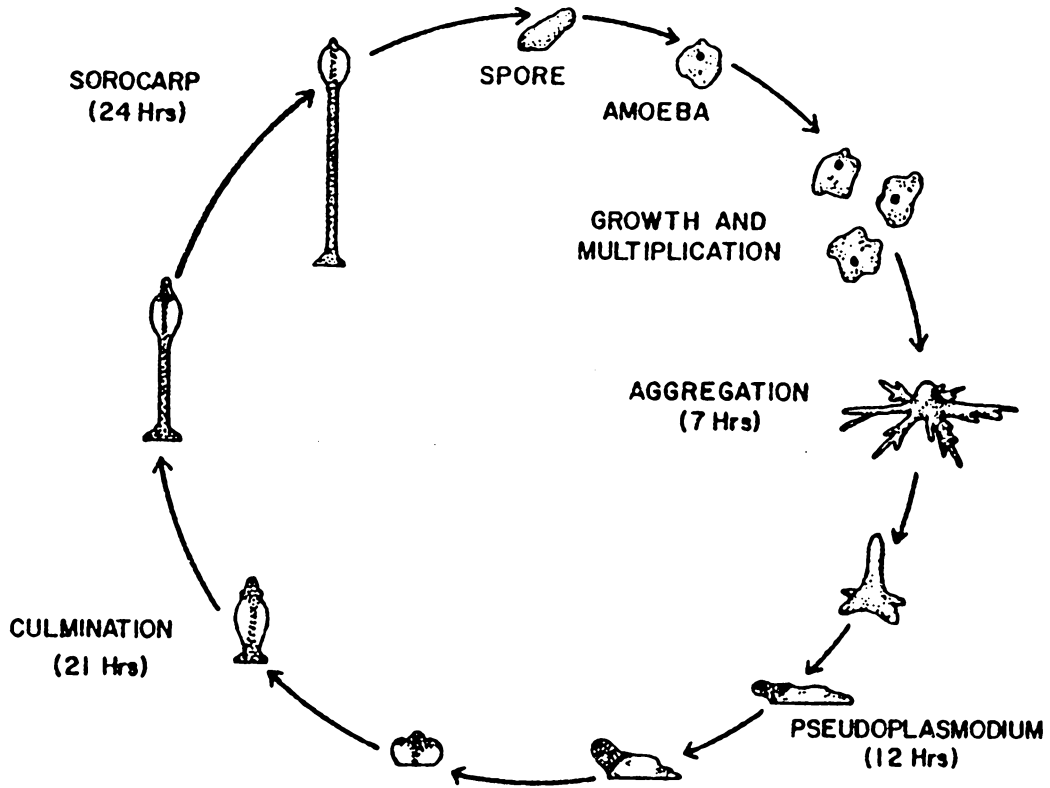


Figure 1. The life cycle of Dictyostelium discoideum.

slug rise up the stalk and differentiate to become spores, each amoeba being eventually encased in an elliptical spore case secreted by the cells (Hohl and Hamamoto, 1969). When favorable conditions are again introduced, the spore cells germinate and begin a new life cycle.

As a model system, Dictyostelium discoideum has many advantages. The life cycle is such that the growth period is separate from the morphogenetic and differentiation processes that follow, simplifying the task of experimental interpretation. In addition, synchronous development can be achieved readily in the laboratory for important biochemical analyses. Despite the small size and apparent simplicity of the organism (with only two differentiated cell types), Dictyostelium discoideum displays multicellular phenomenon similar to metazoans. Assuming there are certain universal laws governing the processes of differentiation and morphogenesis, the study of Dictyostelium development may provide a better understanding of this seemingly complex series of events.

A Proposed Role for Cyclic AMP in Development

Signalling, Chemotaxis, and Adhesion

In a classic experiment, John Bonner (1947, 1949) surrounded a clump of D. discoideum cells by responsive cells and placed them in a gentle stream of water. Since only downstream cells aggregated toward the clump, he concluded that central cells were excreting a soluble chemoattractant molecule. It is widely accepted that adenosine 3':5'-cyclic monophosphate or cyclic AMP (cAMP), was the agent responsible for the observed chemotactic movements. A biochemical understanding of cAMP-involvement in D. discoideum signalling and chemotaxis is an active area of research, the details of which can be found elsewhere (reviewed by Loomis, 1975). As the food source becomes scarce, the cells secrete the cAMP degradative enzyme, phosphodiesterase. When nutrients are depleted, the amoebae secrete a heat-stable molecule which specifically inhibits this enzyme activity (Gerisch et al., 1972). The initiation of normal development is characterized by the resultant increase in cAMP concentration. An extracellular gradient in cAMP is established when a few cells develop the capacity to secrete pulses of cyclic AMP autonomously. Plasma membrane proteins of surrounding aggregation-competent cells are able to bind

this extracellular cAMP (Malchow and Gerisch, 1974; Henderson, 1975), and elicit a fast, but transient activation of adenylate cyclase (Roos et al., 1977), leading to a spike of intracellular cAMP. Since intracellular cAMP peaks coincide tightly with peaks of light-scattering changes (which indicate forward cell movement), Gerisch and Wick (1975) suggest that chemotaxis of surrounding cells reflects a response to the internal rather than external cAMP pool. After a period of increased cAMP synthesis and trans-membrane transport (Robertson et al., 1972), these cells, in turn, secrete periodic pulses of newly-synthesized cyclic AMP (Schaffer, 1976). The signal is thus relayed outward, and cells stream toward the center of the aggregation territory eventually to form a pseudoplasmodium, or slug. It is during the early streaming stage that the first cell-cell contacts are made. Even prior to this stage, though, cAMP has induced cell polarity, with cohesion being localized at the ends of elongated cells to effect the observed inward streaming patterns. Toward a better understanding of cell-patterning, it has been important to elucidate the nature of these first and subsequent cell contacts. What are the aggregation-specific determinates? What biochemical changes are necessarily imposed when a cell interacts with another cell? What is the sequence of events that dictate the changeover from a group of individual cells to an integrated multicellular or-

ganism? Finally, what does cyclic AMP have to do with this progression?

Morphogenesis and Differentiation

Cyclic AMP has been implicated for both the early morphogenetic movements of D. discoideum cells, and subsequent differentiation. Though the exact relationship between these two processes is not well-understood, it appears that, under certain conditions, normal morphogenesis is not essential for cell differentiation. This conclusion has been drawn from experiments using developmental mutants, and from experimental perturbations of normal morphogenesis. Each will be discussed.

Godfrey and Sussman (1982) have written an excellent review dealing specifically with Dictyostelium discoideum developmental genetics. In it they outline the availability and use of developmentally interesting mutants. At present there are mutants affected at every stage of the life cycle, including spore germination, vegetative growth, cell aggregation, slug migration, fruiting body construction, and macrocyst formation. The goal behind isolation of certain of these mutants has been the establishment of systems in which the processes of morphogenesis and differentiation can be studied separately. As a result, factors active in cell-cell communication can more easily be identified and studied

(Abe et al., 1981). Progress is being made in the study of cell cohesion, for instance, through the use of cohesion-defective mutants. Sussman (University of Pittsburgh) described a temperature-sensitive mutant demonstrating normal cohesion properties during aggregation at the restrictive temperature, but loss of cohesion at a time when slugs would form in the wild type (Kay, 1981). Since a 95,000 MW slug membrane glycoprotein described by Steinemann and Parish (1980) was absent in these mutants, it was suggested that this protein may be primarily responsible for post-aggregation cell-cell cohesion.

The effects of nanomolar pulses of cAMP on stimulating normal expression of certain aggregation-specific genes (Darmon et al., 1975; Gerisch et al., 1975) can be mimicked by the continuous application of much higher concentrations of this cyclic nucleotide (Klein, 1975; Sampson et al., 1978). Although normal morphogenesis is blocked, both stalk and spore differentiation can occur (Bonner; 1970, Town et al., 1976; Kay et al., 1978). Stalk differentiation requires cAMP and a low MW diffusible factor (Town et al., 1976; Town and Stanford, 1979). Spore formation requires cAMP (Kay et al., 1978; Kay, 1979) and some other type of cell interaction. Ammonia inhibits the formation of both cell types (Kay, 1979), and acts as a reversible inhibitor of intracellular and extracellular cAMP accumulation (Schin-

dler and Sussman, 1979). Several authors suggest that small diffusible molecules such as cAMP and ammonia may play a role in the establishment of cell pattern.

Many experiments have been devised to show that cells sort out after aggregation (reviewed by Takeuchi et al., 1977). The details of this process, however, are only partly known. Three mechanisms for cell sorting have been proposed and are in the process of being tested. The first proposes chemotactic attraction of cells to a diffusible chemical substance such as cyclic AMP. A second hypothesis involves differential adhesion of prestalk and prespore cells. To distinguish between these first two possibilities, cellular interactions are often studied under circumstances that do not permit the development or maintenance of chemotactic gradients, as in fast-shaken suspension cultures. Still a third proposal suggests differential rates of migration in the slug, so that faster (prestalk) cells end up in front.

A number of experiments tend to support the first hypothesis. If cell masses are surrounded by agar containing cAMP, for instance, only prestalk cells accumulate in the periphery (Matsukuma and Durston, 1979; Sternfeld and David, 1981). When preaggregative cells are separated on density gradients, prespore-preferring cells are found to be more responsive to cAMP than prestalk cells. Discre-

tion should be used, however, in interpretations of these experiments. The role of cAMP in cell sorting may involve more than differential chemotaxis; a cAMP gradient could conceivably have secondary effects on cells which might direct their orientation (Morrisey, 1983).

In favor of the differential adhesion hypothesis, aggregates formed in suspension culture differentiate into a random distribution of prespore and prestalk cells but later organize themselves in a distinct spatial arrangement; prestalk cells partially or wholly surround prespore cells (Forman and Garrod, 1977; Tasaka and Takeuchi, 1981). When disaggregated slug cells are allowed to reaggregate in suspension, though, prestalk cells sort to the inside (Tasaka and Takeuchi, 1979). Clearly, more research is needed before the issue is finally resolved. At present there is evidence in favor of both differential adhesion and chemotaxis. Kopachik (1982) suggests that prestalk cells may be better able to generate a gradient of cAMP (due to increased levels of the cAMP degradative enzyme phosphodiesterase), and therefore begin chemotaxis earlier than prespore cells in a developing slug.

The sorting out process alone may not explain the correct proportioning of the culminating individual. Two mutants have been described, one which forms a stalkless

fruiting structure, and the other which constructs only a long, slender stalk. In each case, the proportion of "prespore" and "prestalk" cells identified at the slug stage was identical to the wild type (Morrissey et al., 1981). Evidently, there are underlying events following sorting out that are necessary to complete the developmental program.

An understanding of pattern formation requires both knowledge of the factors that control differentiation and the elements responsible for their spatial arrangement (Town et al., 1976). Cyclic AMP levels are differentially distributed within the developing organism, and various models of differentiation incorporate this fact into proposed schemes of pattern formation. At the slug stage, cyclic AMP levels are highest in the anterior tip (Rubin, 1976; Bonner, 1949), which apparently functions as an organizer (Rubin and Robertson, 1975). A gradient of cAMP could conceivably be maintained if adenylate cyclase and phosphodiesterase activities (the cAMP-synthetic and degradative enzymes, respectively) were antagonistically distributed within developing individuals. Rutherford et al. (1982a) found that this enzymatic potential does indeed exist. Using ultrasensitive micromethods, they found that during culmination adenylate cyclase was active in prespore cells while phosphodiesterase was active in stalk cells. Activity of these enzymes dropped precipitously in the direction of the opposite cell

type, supporting the proposed existence of a cAMP "source" and "sink". A gradient of cAMP may explain the development of alternate pathways of biochemical differentiation. It may also explain the coordinated forward movement of slugs.

Though it is still unclear whether the developmental fate of a cell is specified according to its position within the cell mass, studies continue to implicate cell-cell contact and cAMP as normal prerequisites for differentiation. With this perspective in mind, the relationship between cAMP and gene activity is explored in the next section.

Gene Activity

The onset of multicellularity in Dictyostelium discoideum occurs about halfway through the developmental program. Though relatively few morphological changes are evident at this time, it is nevertheless a critical and dynamic period of biochemical differentiation; the stage must be set, so to speak, before spore and stalk cell differentiation can proceed.

At a time when cells first make tight contacts (approximately 13 hours into development), pronounced changes occur in the rates of synthesis and accumulation of many proteins and mRNAs (Alton and Lodish, 1977; Williams and Lloyd, 1979; Blumberg and Lodish, 1980). Pulse-labeling with [³⁵S]-methionine reveals that a new class of proteins is

synthesized at this time. Initiation of their synthesis is largely regulated at the level of transcription; of the approximately 3000 new polyadenylated RNA transcripts appearing in the cytoplasm and on the polysomes at this developmental stage (Blumberg and Lodish, 1980), most are not detectable in nuclear RNA from growing (vegetative) cells. Cyclic AMP has been found to be directly involved in controlling this synthesis (Takemoto et al., 1978; Town and Gross, 1978; Landfear and Lodish, 1980).

Once initiated, continued synthesis of these proteins depends on maintenance of cell contact. When aggregates are disrupted by shaking in buffer containing EDTA, selective arrest or attenuation of aggregation-dependent proteins occurs (Landfear and Lodish, 1980). This is probably due, in part, to a decrease in the stability of corresponding developmentally-regulated mRNAs. Mangiarotti et al. (1982) found that, upon disaggregation of 13-h cells, the levels of aggregation-dependent mRNAs, but not of constitutive mRNAs, dropped precipitously. Preferential mRNA stability may be involved in normal differentiation processes, enabling cells to produce and maintain high levels of mRNAs encoding specialized proteins, without substantially increasing their transcriptional capacity. This type of regulation has been documented in other systems, including Friend cell (Volloch and Housman, 1981), myoblast (Affara et al., 1980), and re-

ticulocyte (Lodish and Small, 1976) terminal differentiation.

The stability of aggregation-specific mRNAs, with loss of cell contact, can be greatly improved with addition of cAMP. Even several hours after disaggregation, the levels of most late mRNAs can be maintained in the presence of 10-100 μ M cAMP (Landfear and Lodish, 1980). When cells have been disaggregated for several hours (so that existent aggregation-specific mRNAs are largely degraded), a restoration of the level of most late mRNAs occurs within 3 hours after addition of cAMP (Chung *et al.*, 1981). Although a mechanism of action is unknown, the rapid increase of cAMP levels occurring at late aggregation (Bonner *et al.*, 1969; Malkinson and Ashworth, 1973; Brenner, 1978; Pahllic and Rutherford, 1979) supports a proposed direct role for cAMP in gene expression.

Regulation of Cyclic AMP

The enzymes which regulate cAMP levels, adenylate cyclase and phosphodiesterase (the synthetic and degradative enzymes, respectively), also show stage-dependent activity. An understanding of their placement, activity, and control is prerequisite to an understanding of cAMP regulation.

Adenylate cyclase activity increases sharply at aggregation, coincident with the increase in cAMP (Pahllic and

Rutherford, 1979; Rossomondo and Hesla, 1976; Klein, 1977; Roos et al., 1977). In Dictyostelium, regulation of this membrane-bound enzyme is not well understood. It is not known, for instance, whether it is comprised of three distinct protein components as are found in mammalian systems: a catalytic subunit, regulatory subunit, and hormone receptor (Ross and Gilman, 1982) although, like the mammalian catalytic component, adenylate cyclase activity has been shown to require a divalent cation for activity (Merkle and Rutherford, in preparation). A Dictyostelium GTP-binding protein which serves as a substrate for cholera-toxin catalyzed ADP-ribosylation was recently reported by Leichtling et al. (1981 a,b). Whether this is equivalent to the G/F regulatory component of mammalian adenylate cyclase will necessarily await demonstration of an adenylate-cyclase associated G/F activity in this organism.

It is possible that adenylate cyclase may be regulated by another mechanism. Klein and Darmon (1979) found, for instance, that Dictyostelium adenylate cyclase was stimulated by its product cyclic AMP. Whether cAMP was applied to whole cells constantly or in pulses, it was found that the cAMP-elicited increases in adenylate cyclase activity were transitory, of a few minutes duration. After cAMP was removed, a certain amount of time (refractory period) was required before adenylate cyclase could again be activated to

the same extent (Devreotes and Steck, 1979). This sort of transitory increase in adenylate cyclase is reminiscent of hormone-elicited responses in mammalian systems.

If cAMP is indeed acting as a "first messenger", kinase-mediated phosphorylation may provide the mechanism for such rapid and reversible changes. Using a photoaffinity label, Juliani and Klein (1981) found that cAMP induces a change in a cell-surface cAMP receptor, from a component of 45,000 MW to that of 47,000 MW. More recently, Lubs-Haukeness and Klein (1982) observed cAMP-dependent phosphorylation of a 47,000 MW protein in amoebae. From the rapidity of the response and its dependency on cAMP concentration, this protein may be a regulatory molecule in the adenylate-cyclase enzyme complex.

Activity of the cAMP-degradative enzyme, phosphodiesterase (PDE), also increases upon aggregation (Chassy, 1972; Pannbacker and Bravard, 1972; Toorchen and Henderson, 1979). Composed of a catalytic and regulatory subunit (Franke and Kessin, 1981; Orlov *et al.*, 1981), the enzyme is active in developing stalk cells, but inactive in prespore cells due to binding of the regulatory subunit (Rutherford and Brown, 1983).

The biochemical mechanism of cAMP action is just beginning to be understood with respect to developmental processes. Along with an explanation of kinase-mediated processes

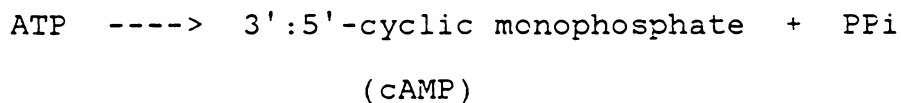
in mammalian systems, this topic is explored in the next section.

Cyclic AMP-Dependent Kinases and Their Actions

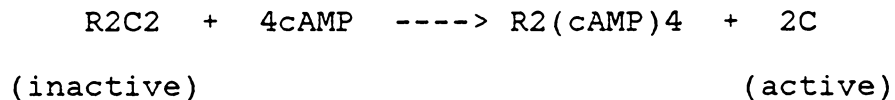
The effects of cAMP on many eukaryotic metabolic pathways and cellular processes are mediated through the activation of protein kinases that catalyze the phosphorylation of seryl and threonyl side chains of many protein substrates (Krebs, 1972; Rubin and Rosen, 1975; Rosen *et al.*, 1977; Cohen, 1978; Krebs and Beavo, 1979).

Two forms of cyclic AMP-dependent protein kinases (cAMPdPK) have been isolated in mammals (Reimann *et al.*, 1971; Rubin *et al.*, 1972), differing on the basis of their elution from DEAE-cellulose. Type I is found predominantly in skeletal muscle, and type II is found in cardiac muscle. Both types consist of two regulatory (R) and two catalytic (C) subunits. The regulatory subunit of cAMPdPK is the major, if not the only receptor known for cAMP in eukaryotes.

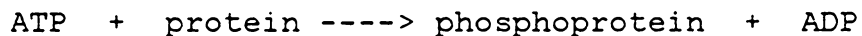
Cyclic AMP-dependent protein kinases have been studied in mammalian systems mainly to gain insight on hormonal regulation. It is known, for instance, that certain membrane-bound hormones activate membrane-bound adenylate cyclase which catalyzes the following reaction:



Cyclic AMP then acts as an allosteric activator of cAMP-dependent kinases. The holoenzyme normally binds four equivalents of cAMP, which causes dissociation of the enzyme into an R-subunit dimer and two monomeric C subunits (Corbin et al., 1978; Builder et al., 1980).



The catalytic subunit, once dissociated, acts as an ATP:protein phosphotransferase.



Protein phosphatases catalyze the reverse reaction (see Krebs and Beavo, 1979).

From characterization of mammalian substrates, it is known that these kinases recognize a phosphorylation site containing two basic amino acid residues, preferably arginines, followed by one or two intervening residues and then a serine (Kemp et al., 1975, 1977; Yeaman et al., 1977). It is the serine residue which provides the actual phosphorylation site. Several substrates have been described for these kinases (see Rubin and Rosen, 1975).

If there could be a key word to bring together the concepts of cAMP-mediated hormonal effects in mammals and the proposed cAMP-mediated effects during Dictyostelium develop-

ment, it might be this: change. Cyclic AMP elicits a rapid change, first by activating cAMP-dependent kinases, which in turn alter the phosphorylation state of certain protein substrates. With respect to hormone stimulation, there is the concept of reversibility; with development, more often the concept of irreversibility. But these are concepts only, based on perceptions of these processes as a whole. Certainly, a molecular understanding of the cAMP-relay system in Dictyostelium discoideum will shed light on part of this complicated cascade of biochemical steps.

Adenylate cyclase, phosphodiesterase, cAMP dependent-kinases, as well as their substrates are important molecules to investigate in order to better understand the role of this cyclic nucleotide in developmental processes. To bring together the concepts of morphogenesis and differentiation, it is important to ask, as Bonner suggests(1974): 1) the nature of these substances and how they are produced, 2) when they are produced, and 3) where they are placed.

The possibility of a cAMP-dependent pathway for phosphorylation in D. discoideum seemed immanent with the reported existence of a cAMP-dependent protein kinase in 2 h starved cells (Sampson, 1977). However, this initial observation was never verified. Indirect evidence for the existence of a cAMP-dependent protein kinase in D. discoideum was then provided by the report of a cAMP-binding protein

(MW=41,000) capable of inhibiting the catalytic subunit of beef heart cAMP-dependent protein kinase (Leichtling et al., 1981 b)

During the course of this study, we reported the existence of a cAMP-dependent protein kinase in partially-purified 110,000 X g soluble extracts of post-aggregation stages of Dictyostelium discoideum (Rutherford et al., 1982 b). The enzyme was activated up to 6-fold by cyclic AMP, and co-purified with a cAMP-binding activity. Partially-purified protein kinase activity found in earlier stages failed to show cAMP dependency, although membrane-bound activity was not investigated. The cAMPdPK that we observed in post-aggregation stages could be reproducibly extracted, providing firm evidence in vitro for cAMP-mediated effects through protein phosphorylation in this organism.

Rationale

Cyclic AMP has been implicated in the controlling processes of morphogenesis and differentiation, and yet a mechanism of action is unknown. The cellular slime mold, Dictyostelium discoideum, provides a convenient model system with which to study these processes at the molecular level. Since the regulatory subunit of cyclic AMP-dependent kinase is the only known receptor for cAMP in other eukaryotic regulatory systems, it is hypothesized that a cAMP-dependent

protein kinase similarly exists in Dictyostelium discoideum. and acts as a post-translational control mechanism analogous to hormonal regulation in mammals. As a chemical transducer for changes in cAMP concentration (both intercellular and extracellular in the case of Dictyostelium discoideum), cAMP-dependent kinase would act at the level of enzyme activity, by catalyzing the transfer of phosphate to developmentally regulated proteins. In a similar way, cyclic AMP (via the kinase) could directly or indirectly control processes at the level of transcription.

In order to substantiate a cAMP-mediated pathway, it is important to show the presence of phosphoproteins which are preferentially phosphorylated in the combined presence of cAMPdPK holoenzyme and cAMP. The goals of this study were to identify the patterns of endogenous phosphorylation during differentiation, and begin a characterization of those phosphoproteins that appear to be cAMP-dependent. Since cAMPdPK has been shown to exist in the cytoplasm, initial emphasis is focused on substrates in this cell compartment. It is not unreasonable to suppose that substrates for the cAMP-dependent kinase also exist in the plasma membrane or within the nucleus.

MATERIALS AND METHODS

Strains, growth conditions, and preparation of tissue

Growth and differentiation of Dictyostelium discoideum amoebae, wild-type strain NC-4, were carried out according to methods outlined by Rutherford (1976). Myxamoebae were grown from spores in two-membered culture with Escherichia coli B/r on sheets of nutrient agar. As amoebae began to clear the culture of bacteria (approximately 48 h growth at 23°C), they were washed off the agar in ice-cold Bonner's salts solution (Bonner, 1947), and collected by centrifugation at 500 X g for 3 min. The cells were then washed twice in cold Bonner's salts to remove remaining bacteria and spread on non-nutrient agar sheets. At the desired stage of development, cells were removed with cold distilled water, pelleted by low-speed centrifugation at 500 X g for one minute, and resuspended (20 volumes/ml packed cells) in cold TAMB buffer [50 mM Tris-HCl, pH 7.5, containing 2 mM 2-mercaptoethanol, 0.2 mM benzamidine, and 0.02% sodium azide (Rutherford et al., 1982 b)]. Most preparations contained 6-8 gm wet weight of cells (approximately 200 mg protein), although smaller amounts could be used with similar results. The cells were then evenly distributed by two

strokes of a Potter-Elvehjem tissue grinder (low-speed, on ice), disrupted by three 45 second exposures to a one centimeter probe of a sonic cell disrupter (Model 150, Virtis, Gardiner, N.Y.) at a setting of 80, and centrifuged at 110,000 X g for 60 minutes. Further purification by column chromatography was performed immediately after centrifugation.

Column chromatography

The 110,000 X g supernatant (10-80 ml) was applied to a DE-52 cellulose column (1.6 X 13 cm) and eluted at 80 ml/h with TAMB (pH 7.5). Material that did not bind to the resin (flow-thru) was collected and either assayed individually for cAMPdPK activity and endogenous phosphorylation and/or pooled and concentrated to 1-5 ml (PM 10 membrane, Amicon) for further purification on a Sephacryl S-300 column (1.6 cm X 90 cm at a flow rate of 30 ml/h). For some experiments, S-300 fractions containing cAMPdPK activity were pooled and concentrated prior to assay of endogenous phosphorylating activity.

Separation of the cAMP-dependent holoenzyme into its component regulatory and catalytic subunits was achieved by chromatofocusing with polybuffer (Pharmacia). The 1.5 X 15 cm column was first equilibrated with 25 mM TAMB before loading of the sample (in 50 mM TAMB), then eluted with po-

lybuffer (pH 4.0, Pharmacia) at a flow rate of 30 ml/h. Ten minute fractions were collected. Catalytic activity was assayed as described below, and peak activity typically eluted at approximately pH 5.5. Peak cAMP-binding activity eluted at approximately pH 5.25.

Protein kinase and endogenous phosphorylation assay

Protein kinase activity was assayed according to a modification of a procedure described by Gill and Walton (1979). Enzyme activity was measured as the rate of radioactive phosphate incorporation from [γ - ^{32}P]ATP into acid-insoluble protein. In a total assay volume of 50 μl , 25 μl of sample was added to an equal volume of reaction mix. Reaction mix contained 50 mM potassium phosphate (pH 6.5) or TAMB buffer (pH 7.5), 3 mM dithiothreitol, 10 mM MgCl_2 , 24 μM [γ - ^{32}P]ATP (0.4-1.2 Ci/mmol) and 0.8 mg/ml histone VII-S (Sigma), with and without 20 μM cAMP. After incubation at 23°C for a specified amount of time, a sample of the reaction volume was removed to 1 cm square pieces of filter paper (Whatman 3 MM) and immediately transferred to cold 10% TCA. After 15 minutes, the papers were then subjected to hot 5% TCA (3 min), cold 5% TCA (5 min), and acetone (5 min), before being dried. Radioactivity was determined using a liquid scintillation counter.

In some column fractions, endogenous cAMPdPK activity copurified with Dictyostelium discoideum phosphoproteins. In order to assay the amount of endogenous phosphorylation in these samples, histone VII-S was omitted from the reaction mixture. By doubling the reaction volume, samples could be prepared for both the filter paper assay and gel electrophoresis, as described below.

SDS-polyacrylamide gel electrophoresis and autoradiography

The sample and reaction mixture were allowed to incubate, as described above, then the reaction was terminated by adding 50 ul of stopping solution (9% SDS, 15% glycerol, 0.05% bromophenol blue, 30 mM Tris-HCl, pH 7.8) and boiling for 5 minutes. After cooling to room temperature, 50 ul of dithiothreitol (initial concentration 75 mg/ml) or 8% 2-mercaptoethanol was added. Samples were then subjected to SDS-polyacrylamide slab gel electrophoresis (7 or 10% acrylamide), protein staining and/or autoradiography according to the method of Rudolph and Krueger (1979). Quantitative differences in protein phosphorylation due to cAMP addition were determined using a Quick-Scan, Jr densitometer from Helena Laboratories.

RESULTS

Endogenous phosphorylation at four stages of development

The phosphorylation of endogenous proteins was measured in vitro, at four developmental stages after partial purification of 110,000 X g soluble extracts. Since cAMPdPK activity was previously demonstrated in the flow-thru fractions of a DE-52 anion exchange column (Rutherford et al., 1982 b), it was thought that possible substrates for this enzyme may similarly be found in these partially purified soluble extracts. Endogenous phosphorylation was assayed as described in Materials and Methods, and the results of two separate experiments demonstrated similar trends. Figure 2A shows endogenous phosphoprotein patterns of pooled DE-52 cellulose flow-thru fractions, in the presence and absence of cAMP, following SDS-polyacrylamide gel electrophoresis and autoradiography. The tissue was prepared in the same way for each of four developmental stages: early aggregation, late aggregation (with tips), late slug, and culmination. Marked differences in endogenous phosphorylation appeared to characterize the developmental process. At the early aggregation stage (not shown), no endogenous phosphorylation was observed. Likewise, there was little phospho-




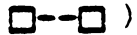
rylation at the late aggregation stage, except for a band at approximately 53,000 MW which showed distinct labeling at this stage. However, at the slug stage, at least 20 phosphoproteins were labeled. This sudden increase in the number of proteins phosphorylated in vitro correlated with a coincident appearance of cAMP-dependent protein kinase at this stage (Rutherford et al., 1982). The culmination stage showed phosphate incorporation into some of the same proteins that were labeled at the slug stage. Although there were phosphoproteins which were labeled more intensely at this stage than at the slug stage (a band at approximately 107,000 MW, for instance), in general the proteins were labeled less intensely. The marked stage-dependent differences in endogenous phosphorylation were accompanied by only minor differences in total protein, as revealed by Coomassie staining (Fig. 2B).

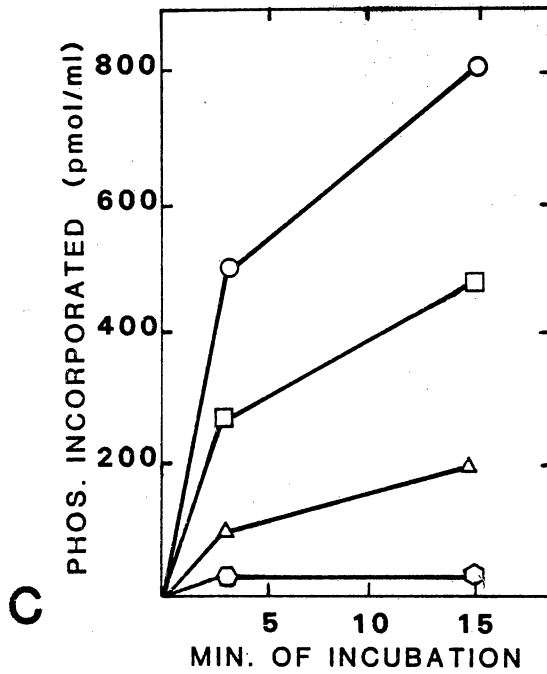
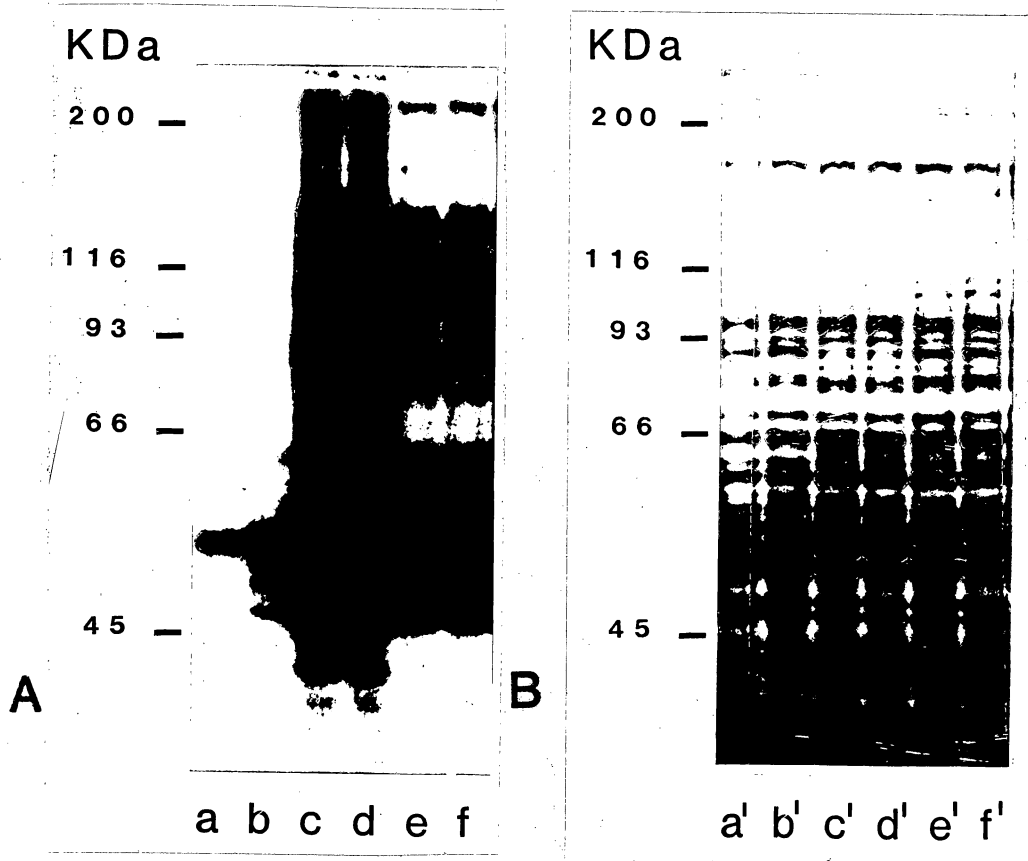
Although the presence of cAMP-dependent kinase was confirmed in these post-aggregation samples (using commercial histone VII-S as substrate), no dependency was noted in this stage study. A more detailed analysis of the time course of phosphorylation, however, showed that under the conditions of this assay, endogenous substrates for the cAMP dependent kinase were maximally labeled in less than 15 minutes of incubation (see Fig. 7, below), even in the absence of cAMP.

Figure 2. Stage-dependent phosphorylation of DE-52 flow-thru fractions after incubation with 10 μ M [γ - 32 P] ATP.

A. Autoradiograph of SDS-polyacrylamide gel, showing soluble 32 P-labeled phosphoproteins from: late aggregation with tips (a and b), late slug (c and d), and culmination (e and f) tissue. Endogenous phosphorylation of early aggregation tissue (not shown) indicated negligible phosphate incorporation into DE-52 soluble flow-thru proteins. Samples were incubated 15 minutes at 23°C in the presence (a,c,e) and absence (b,d,f) of 20 μ M cAMP. The migration of standard marker proteins is indicated on the left.

B. Coomassie stain (a'-f') of corresponding samples shown in Fig. 2A.

C. Time course of phosphate incorporation into total protein from pooled DE-52 flow-thru fractions, in the absence of cAMP: early aggregation (); late aggregation with tips (); late slug (); and culmination ().



Total endogenous phosphorylation activity for the four stages of development (as assayed by TCA precipitation of DE-52 flow thru samples onto filter paper, see Materials and Methods) was measured after 3 min and 15 min incubation times (Fig. 2C). The results were similar to those observed with SDS-PAGE : low levels of phosphorylation were found in the early stages of development, with a peak of activity occurring at the late slug stage.

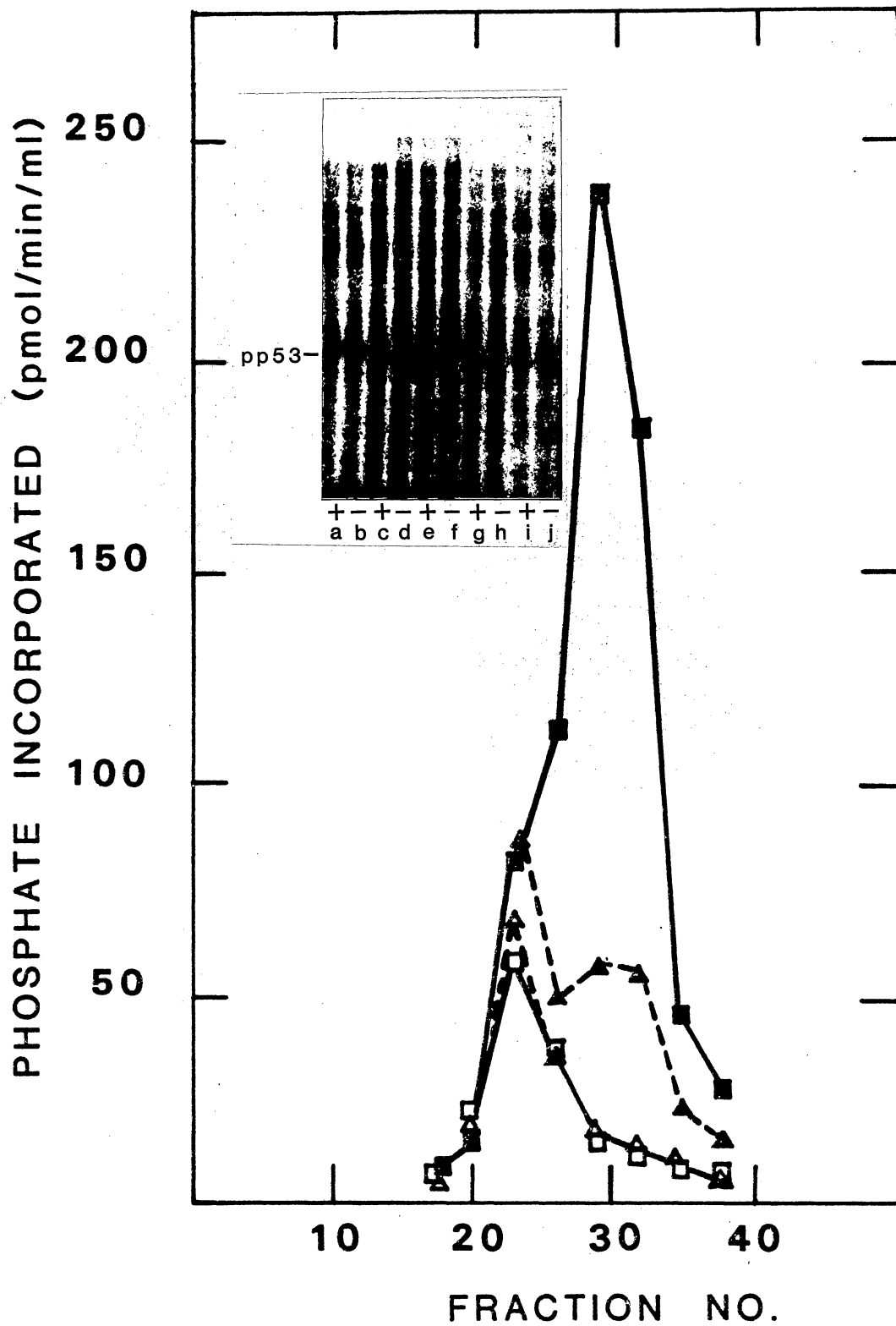
Endogenous phosphorylation and cAMPdPK activities of soluble fractions from S-300 purified slug tissue

When DE-52 flow-thru proteins from the early slug stage were separated by S-300 Sephacryl gel filtration (Fig. 3), endogenous phosphorylation (filter paper assay) consistently showed a single peak corresponding to the void volume of the column. Protein kinase showed two peaks of activity (with histone VII-S as substrate): a cAMP independent activity in the void volume (coincident with the total endogenous phosphorylation peak), and a second cAMP dependent peak which eluted at an approximate molecular weight of 170,000. When the void volume was assayed for endogenous phosphorylation (SDS-PAGE followed by autoradiography) a major protein (MW 53,000) was labeled (see insert, Fig. 3). The presence of 20 uM cAMP did not increase the amount of phosphate incorporated. Therefore, the 53,000 MW phosphoprotein (pp53) did not appear to be a substrate for the cAMPdPK. Additional

Figure 3. Endogenous phosphorylation and cAMPdPK activities of soluble fractions from slug tissue, following Sephacryl S-300 purification step.

Samples were incubated with 10 μM [γ - ^{32}P] ATP for 15 min at 23°C. The solid symbols show an assay for cAMPdPK in which the commercial substrate, histone VII-S (Sigma, 0.4 mg/ml), was included in reaction mixture with (■—■) and without (▲—▲) 20 μM cAMP. The open symbols show an assay for phosphate incorporation into total endogenous protein in the presence (△—△) and absence (□—□) of 20 μM cAMP.

The insert shows a major phosphorylated band (MW=53,000) observed in consecutive void volume fractions. Samples of fraction 21 (a,b), 22 (c,d), 23 (e,f), 24 (g,h), and 25 (i,j) were incubated with 10 μM [γ - ^{32}P] ATP for 3 min at 23°C, in the presence (+) and absence (-) of 20 μM cAMP.



evidence that this protein could not be phosphorylated by cAMPdPK came from a single experiment in which fractions 22 and 30 from the S-300 were mixed and incubated in the absence of cAMP (Fig. 4). The rate of total endogenous phosphorylation was not changed by increased amounts of cAMPdPK.

Demonstration of cAMP-dependent phosphorylation of endogenous substrates

Although substrates for cAMPdPK could not be demonstrated in whole cell extracts assayed in vitro, further purification of the kinase activity revealed their presence. In S-300 fractions containing cAMPdPK activity (see Fig. 3), phosphate incorporation into total endogenous substrates appeared negligible relative to that for added histone VII-S (0.8 mg/ml) However, if these fractions were pooled and concentrated 10-20 fold, an increase to measurable levels of cAMP-dependent endogenous phosphorylation was observed at the late slug stage (Table 1). Aggregation tissue prepared in the same way showed no endogenous cAMP-dependent phosphorylation activity. This is again in agreement with our previous report that cytosolic cAMPdPK appears only after tip formation at tight aggregation (Rutherford et al., 1982). Cyclic AMP-dependent endogenous phosphorylation could also be observed when concentrated S-300 fractions containing cAMPdPK were incubated with [γ - 32 P]ATP then prepared for gel electrophoresis and autoradiography. Cyclic AMP-dependent

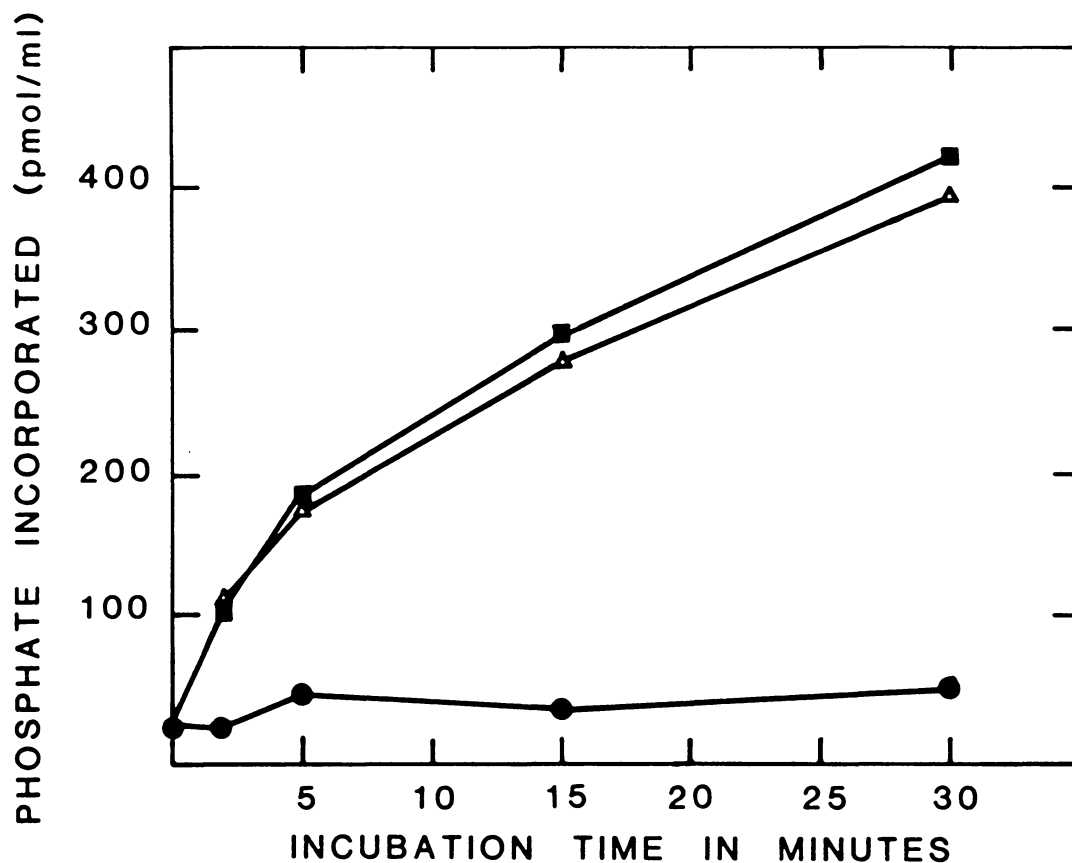


Figure 4. Time course of phosphate-incorporation into TCA-precipitable material from void-volume (cAMP-independent) and peak cAMPdPK S-300 fractions during incubation with 10 uM [γ - 32 P] ATP.

Fractions 30 (●--●) and 22 (■--■) were incubated alone and together (Δ--Δ) at 23°C, in the absence of cAMP.

Table 1. Total endogenous phosphorylation and cyclic AMP-dependent protein kinase activities in S-300 partially purified soluble fractions of aggregation and slug tissue.

Developmental Stage	With (+) or Without (-) Histone VII-S	With (+) or Without (-) cAMP	Phosphate Incorporated (pmol/min/ml)
Aggregation	-	+	5.8
	-	-	6.0
	+	+	25
	+	-	25
Slug	-	+	90
	-	-	54
	+	+	160
	+	-	92

Fractions #25-35 from an Sephadex S-300 column were pooled and concentrated 10-fold for each stage. Samples were then incubated with [γ - 32 P] ATP for 2 min with (+) and without (-) 20 uM cAMP and/or histone VII-S (Sigma) at 23°C.

Figure 5. Endogenous cyclic AMP-dependent phosphate incorporation into soluble proteins after S-300 chromatography.

Autoradiograph of SDS-polyacrylamide gel, showing phosphate-labeled soluble phosphoproteins from slug tissue. Fractions containing cAMPdPK after S-300 chromatography were pooled and concentrated 10-fold, then incubated with 10 μM [γ - ^{32}P] ATP for 5 min at 23°C in the presence (a,+) and absence (b,-) of 20 μM cAMP. The apparent molecular weights of five phosphorylated bands are shown on the left.

KDa

pp₁ 107 —
pp₁ 91 —

pp 75 —
pp 69 —



+ -
a b

bands were observed with molecular weights of 110 KDa, 107 KDa, 91 KDa, 75 KDa, and 69 KDa (Fig. 5 and 6).

The time of development, degree of growth synchrony, incubation time in vitro, gel concentration, and exposure time to X-ray sensitive film all determined which dependent proteins were observed. The bands migrating at approximately 107 KDa and 91 KDa were observed in ten experiments involving early-to-late slug tissue. A higher molecular weight cyclic AMP-dependent phosphoprotein (>200 KDa) also appeared in these extracts from the slug stage, but could only be visualized when low (7-8%) gel concentrations were used.

A time course for total phosphate incorporation into S-300 endogenous substrates showed an increase in labeling with incubation times up to 5 minutes (Fig. 7A). The addition of 20 μ M cAMP in the reaction mixture increased the amount of phosphate incorporated by approximately 40%. Longer incubation times showed a leveling off of phosphate incorporated. In a parallel assay, specific endogenous phosphoproteins were separated by SDS-PAGE and visualized by autoradiography (Fig. 7B). An increase in phosphate incorporation with time of incubation and a marked cyclic AMP-dependent phosphorylation of bands at 107,000 and 91,000 MW were noted. Cyclic AMP dependency was most easily visualized on autoradiographs for the shorter incubation times. Lanes c and d of Fig. 7B, for instance, showed clear cAMP dependency

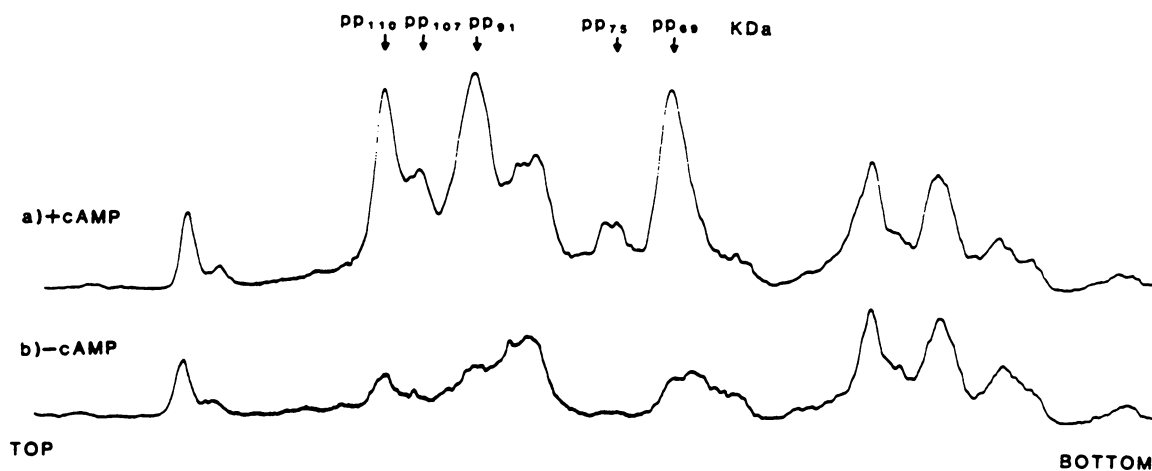


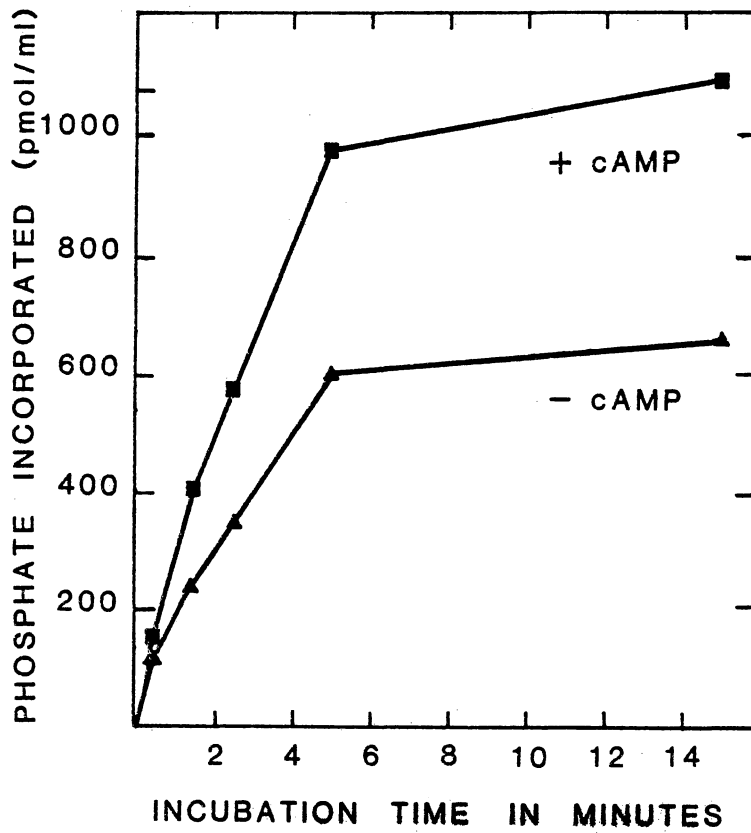
Figure 6. Densitometer scans of autoradiograph (lanes a and b) shown in Fig. 5.

Bands which appear to be cAMP-dependent are indicated with arrows.

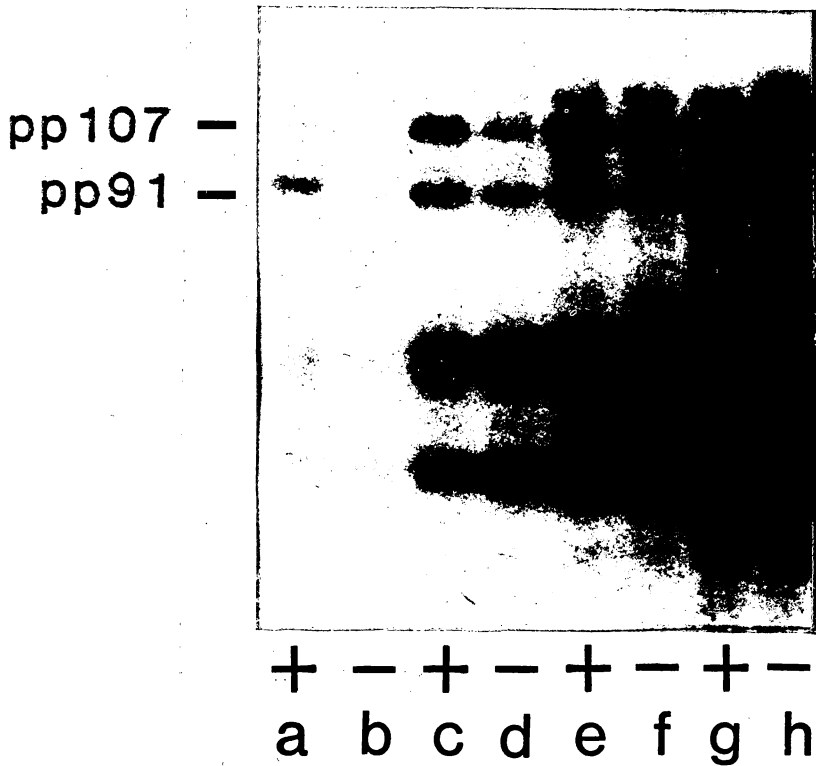
Figure 7. Time course and cAMP dependency of phosphate-incorporation into soluble proteins from slug tissue, after Sephacryl S-300 chromatography.

A. Time course of radioactive phosphate incorporation into total TCA-precipitable material. Pooled and concentrated S-300 soluble fractions containing cAMPdPK activity were incubated at 23°C with 10 uM [γ - 32 P] ATP in the presence (+) and absence (-) of 20 uM cAMP.

B. Autoradiograph of SDS-polyacrylamide gel showing pooled and concentrated S-300 fractions containing cAMPdPK activity after incubation with 10 uM [γ - 32 P] ATP for 1 min (a and b), 3 min (c and d), 5 min (e and f), and 15 min (g and h) in the presence (+) and absence (-) of 20 uM cAMP.



A



B

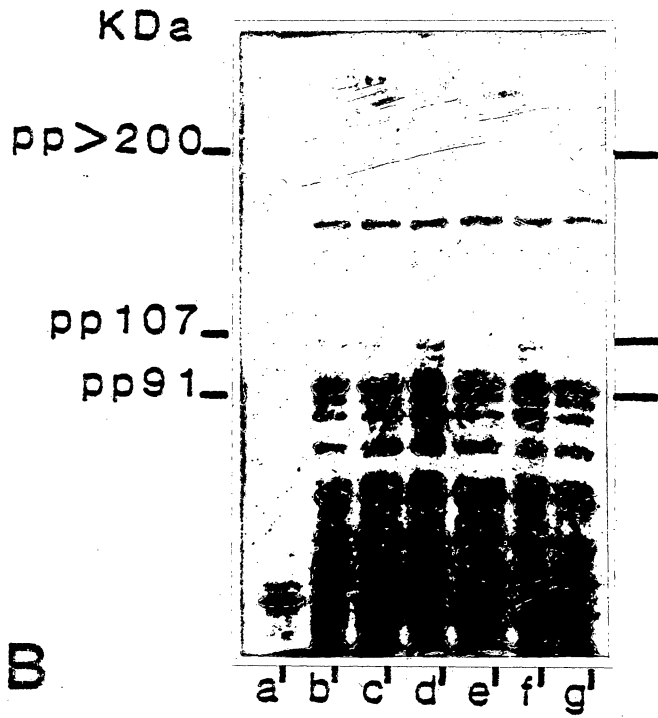
with an incubation time of 3 min. For incubation times greater than 15 min (lanes g and h), the dependency was not as marked. Apparently the endogenous Dictyostelium cAMPdPK (or another kinase?) phosphorylated these proteins, even in the absence of cAMP. The highest MW cAMP-dependent band (>200,000) observed only with low gel concentrations, showed a similar increase in phosphate incorporation with incubation time (not shown). Neither total endogenous phosphorylation nor specific endogenous phosphorylation was affected by micromolar concentrations of 5'AMP (data not shown).

Upon further purification of cAMPdPK activity by chromatofocusing, the enzyme dissociated into catalytic and regulatory subunits. In two separate experiments, we tested whether this low MW catalytic preparation would phosphorylate the same proteins as the holoenzyme form. Concentrated S-300 fractions containing endogenous activity were heated (60°C for 10 min, or boiling for 1 min), then mixed with the catalytic subunit. In each case, the catalytic subunit alone showed no endogenous activity (Fig. 8A), indicating that the substrates separated from the enzyme during chromatofocusing. Likewise, the heated S-300 fractions showed no endogenous phosphorylating activity. When the two were mixed, however, phosphorylation of both pp91 and ppl07 occurred, with the latter showing the greater extent of phosphorylation. Thus the free catalytic subunit showed the same

Figure 8. Phosphorylation of pp 107 and pp 91 in heat-treated S-300 fractions by partially-purified Dictyostelium cAMPdPK catalytic subunit. Catalytic subunit was obtained after chromatofocusing of a partially-purified cAMP-dependent holoenzyme (see Materials and Methods).

A. Autoradiograph of SDS-polyacrylamide gel, showing phosphate incorporation after 2 minute incubation with 10 μM [γ - ^{32}P]ATP at 23°C: (a) partially-purified Dictyostelium catalytic subunit alone, (b) concentrated S-300 sample in the presence of 20 μM cAMP, (c) concentrated S-300 sample in the absence of cAMP, (d) concentrated S-300 sample, prepared by heating 10 min at 60°C prior to assay, with 20 μM cAMP, (e) same as "d", except with added partially-purified catalytic subunit preparation (f) concentrated S-300 sample, prepared by boiling 1 min prior to assay, with 20 μM cAMP. (g) same as "f", except with added partially purified catalytic subunit preparation.

B. Coomassie stain (a'-g') of corresponding samples shown in Fig. 8A.



specificity for the phosphorylation of these proteins as did the holoenzyme. Fig. 8B shows the protein pattern for the samples presented in Fig. 8A.

DISCUSSION

The evidence reported here for cytosolic cAMP-dependent phosphorylated substrates in Dictyostelium discoideum confirm and extend our earlier report of a potential cAMP-mediated pathway during later development of this organism. It is interesting that the only major change in the pattern of Dictyostelium discoideum gene expression occurs at some time between the end of the early aggregation stage and the appearance of tips on (13 h) multicellular aggregates. It is during this period that approximately 3000 new polyadenylated RNA species first appear, coincident with a peak of intracellular cAMP (Blumberg and Lodish, 1980), and immediately preceding the onset of spore and stalk cell differentiation. Continued synthesis of these aggregation-specific proteins apparently depends on continued cell-cell interaction. When cell aggregates are dispersed, synthesis of 45 aggregation-specific polypeptides ceases (Alton and Lodish, 1977). Likewise, when disaggregated cells of Dictyostelium pseudoplasmodia are incubated with shaking in a buffer containing EDTA, the activity of several developmentally regulated enzymes decreases rapidly (Okamoto and Takeuchi, 1976; Newell et al., 1971, 1972; Gross et al.,

1977). The nature of the "developmental switch" which initiates transcription of aggregation-specific polypeptides is unknown. However, exogenous cAMP has been shown to have a direct effect on gene expression and may maintain the synthesis of these proteins in disaggregated cells by stabilizing their corresponding messenger RNAs (Chung et al., 1982). A biochemical understanding of this process is presently being sought.

The recent discovery of a soluble cAMP-dependent protein kinase in post-aggregation stages of Dictyostelium discoideum (Rutherford et al., 1982; Leichtling et al., 1982; Cooper et al., 1982; de Gunzburg and Veron, 1982) supports the hypothesis that cAMP-dependent protein phosphorylation may be an important mechanism of developmental control processes, including those associated with differentiation and cell patterning. In this study, further support is given to this idea. Six cAMP-dependent cytosolic phosphoproteins are described, with apparent subunit molecular weights of >200,000, 110,000, 107,000, 91,000, 75,000, and 69,000. Additional endogenous substrates may exist in cytosolic fractions other than those tested. Using this purification scheme, cyclic AMP dependency of phosphoproteins was first discerned at the slug stage, and the relative amount and/or degree of phosphorylation changed markedly with developmental stage. Pre-aggregation tissue contained negligible amounts of endogenous cAMP dependent phosphorylation activity.

Cyclic AMP has previously been linked to in vivo changes in the pattern of Dictyostelium phosphoproteins during development. Using a ^{32}P labeling procedure, Coffman et al. (1981) showed, for instance, that the addition of cAMP caused the premature disappearance of four major phosphoproteins in vivo with apparent subunit molecular weights of 34,000, 38,000, 47,000 and 50,000. Two membranal proteins, with apparent subunit molecular weights of 80,000 (pp80) and 81,000(pp81) appeared precociously in response to added cAMP. In at least one instance (pp80), cAMP was shown to stimulate the synthesis of the phosphoprotein (Coffman et al., 1982). Lubs-Haukeness and Klein (1982) reported a 47,000 MW protein, possibly a membrane-bound cAMP binding protein, that was phosphorylated in the presence of cAMP. Finally, Malchow et al., (1981) showed that chemotactic stimulation of Dictyostelium cells by cAMP increased the incorporation of ^{32}P into myosin heavy chains (MW 210 KDa), a process which was determined to be calmodulin and calcium-dependent.

Cyclic AMP-dependent phosphorylation of Dictyostelium proteins in vitro, using [^{-32}P]ATP as a phosphate donor, has not previously been reported, probably because of past difficulties in purifying and assessing cAMPdPK activity.

In axenic strains, radioactive inorganic phosphate may be introduced to cell cultures with the result that endoge-

nous pools of [γ - ^{32}P]ATP are formed. This is the basis for a common in vivo assay for protein phosphorylation. The labeled phosphate is transferred to potential endogenous substrates of cAMP-dependent protein kinase. Phosphate incorporation into protein at various developmental stages (with subsequent partial purification) is now being studied to ascertain possible in vivo significance. For now, it can be concluded that several endogenous substrates for cAMPdPK exist in the soluble fraction of post-aggregation cells of Dictyostelium discoideum. It will be interesting to determine 1) if these substrates are associated with organelles or other cytosolic structures, 2) the tissue specificity of these substrates, and 3) a possible relationship to known cAMP-mediated effects concerned with control of gene expression, differentiation, and cell patterning.

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ABSTRACT

ENDOGENOUS CYCLIC AMP-DEPENDENT PHOSPHORYLATION

IN VITRO

OF CYTOSOLIC PROTEINS FROM

DICTYOSTELIUM DISCOIDEUM

by

Lynn Teresa Frame

Endogenous phosphorylation was measured in soluble fractions at four stages of Dictyostelium discoideum development. A peak of activity occurred at the slug stage, coincident with the appearance of cyclic AMP-dependent protein kinase. After partial purification by DE-52 cellulose and Sephacryl S-300 chromatography, cyclic AMP dependency of six cytosolic proteins was observed, with apparent subunit molecular weights of > 200,000, 110,000, 107,000, 91,000, 75,000, and 69,000. Phosphorylated bands at 107,000 and 91,000 MW were found to be specific for a partially-purified catalytic subunit prepared from the Dictyostelium holoenzyme form.