The Relationship Between Two Forms of Glycogen Phosphorylase in *Dictyostelium discoideum*

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(ABSTRACT)

The cellular slime mold, *Dictyostelium discoideum*, provides an ideal model system to study eukaryotic cell differentiation. In *D. discoideum* glycogen degradation provides precursors for the synthesis of developmentally regulated structural end products. The enzyme responsible for glycogen degradation, glycogen phosphorylase, exists in active and inactive forms. The activity of the 'a' form (the active form) is independent of 5'adenosine monophosphate (5'AMP) while the activity of the 'b' form (the inactive form) is 5'AMP dependent. The two forms are developmentally regulated. Polyclonal antibodies raised to the purified forms of this enzyme show low cross reactivity. The anti-'a' antiserum reacts with a 104 kd protein that is associated with phosphorylase 'a' activity; the anti-'b' antiserum reacts with a 92 kd protein that is associated with phosphorylase 'b' activity and cross reacts weakly with the 104 kd protein. Cyclic AMP perturbation of intact cells caused induction of both phosphorylase 'a' activity and the appearence of the 104 kd protein. Immunotitration data suggest that the 'a' form accumulates due to *de novo* protein synthesis, although this result must be interpreted with caution. *In vitro* translation experiments indicate that separate mRNA species exist for the two forms of phosphorylase. The mRNA for the 'b' form is present throughout development while that of the 'a' form appears late in development.

Acknowledgements

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1.0 Introduction

The developmental process of multicellular organisms involves a remarkable and complex series of events. Obviously, the progression from a unicellular fertilized egg to a complex organism containing many different cell types requires elaborate biochemical control. Thus, central to the understanding of organismic development is the elucidation of the molecular mechanisms that trigger developmental events such as cell differentiation. The elucidation of the molecular basis for organsimic development and cell differentiation is also crucial to the understanding of aging. Also, developmental anomalies such as cancer and birth defects can only be understood if the normal regulatory mechanisms of differentiated or differentiating cells are determined. Model systems have contributed immensely, and continue to contribute, to our understanding of the molecular basis for development and cell differentiation. The cellular slime mold, *Dictyostelium discoideum*, provides an ideal model system to study the processes involved in cell differentiation and organismic development. Many aspects of *D. discoideum* make it very suitable as a model system; this organism has a short, simple, and well documented developmental cycle, is easily cultured in the laboratory, and can readily be induced to undergo development.

1.1 The Life Cycle of Dictyostelium discoideum

The life cycle of *Dictyostelium discoideum* is shown in Figure 1. *D. discoideum* was first isolated and characterized by Raper (1935). Vegetative cells of *D. discoideum* are ameboid in appearance and are found living in the soil of most temperate forests; in this environment they feed on bacteria and particulate organic matter. When the local supply of nutrients becomes depleted the behavoir of the amoebae changes dramatically. Individual amoebae begin streaming together, in a chemotactic response to cyclic adenosine monophosphate (cAMP), and form a loose aggregate of cells. This aggregate of cells becomes, approximately 12 hours after the initiation of nutrient depletion, slug like in appearance and is called a pseudoplasmodium, or simply a slug. The slug migrates along the substratum, and secretes a slime sheath. The cells making up the slug are no longer a homogeneous mixture; rather, two distinct cell types are seen. This was first observed by Raper (1940), who noticed that the slug seemed to be composed of two distinct regions of cells, and Bonner (1952), who reported that, in response to vital dyes, the cells making up the anterior portion of the slug exhibited different staining than those in the posterior portion of the slug. Raper and subsequent investigators have shown that the cells at the anterior portion of the slug ultimately form a slender stalk upon which a fruiting body, containing mature spores derived from the posterior portion of the slug, rests (Raper, 1940; Bonner, 1952; Bonner et al., 1955); thus, the anterior-most cells in the slug are called pre-stalk cells and the cells from the posterior region of the slug are called pre-spore cells. The transformation of the slug into a mature fruiting body begins when slug migration ceases and a stage called culmination begins. At the culmination stage, the anterior-most of the pre-stalk cells begin to differentiate into mature stalk cells; these cells begin to secrete cellulose, which is the major component of the stalk, and also begin to form the stalk structure. The stalk continues to elongate due to the movement of stalk cells from the apex to the base of the stalk in what Bonner calls a "reverse fountain" pattern of movement (Bonner, 1944; Raper and Fennell, 1952); the elongation of the stalk ultimately causes the mass of pre-spore cells from the posterior of the slug to be lifted upward. As this lifting occurs the pre-spore cells differ-

entiate into spores and the mature fruiting body is thus formed. Each spore in the fruiting body is capable of developing into an amoeba under suitable conditions. Fruiting bodies are usually seen within 24 hours of the onset of nutrient depletion.

Clearly, *D. discoideum* is an ideal model system to study cell differentiation and development. The developmental cycle is well characterized, rapid, and simple; indeed, this organism represents probably the simplest developmental model that can can be imagined, in which one initial cell type (vegetative amoebae) differentiates into two cell types (stalk cells and spore cells).

1.2 The Role of cAMP in the Developmental Cycle of Dictyostelium discoideum

One of the most important regulatory molecules in all eukaryotic systems is cAMP. Not surprisingly, this molecule has been implicated as an important regulatory agent in several different aspects of the life cycle of *D. discoideum*. As mentioned above, cAMP is the chemoattractant molecule responsible for the aggregation of amoebae; considrerable evidence is present implicating cAMP as the chemoattractant agent (Konijn, et al., 1968; Robertson et al., 1972). Chemotaxis begins when certain amoebae begin secreting wave-like pulses of cAMP. The cAMP impinges on cell surface cAMP receptors of other amoebae, inducing the chemotactic response and activating adenylate cyclase, which results in an increase in intracellular cAMP levels; secretion of intracellular cAMP by these amoebae amplifies the chemotactic signal (Shaffer, 1975; Roos et al., 1975; Gerisch and Wick, 1975), and aggregation eventually results. cAMP levels are modulated by cAMP phosphodiesterase activity; three different phosphodiesterase enzymes (intracellular, membrane bound, and extracellular) appear to be present in *D. discoideum* (Devreotes, 1982).

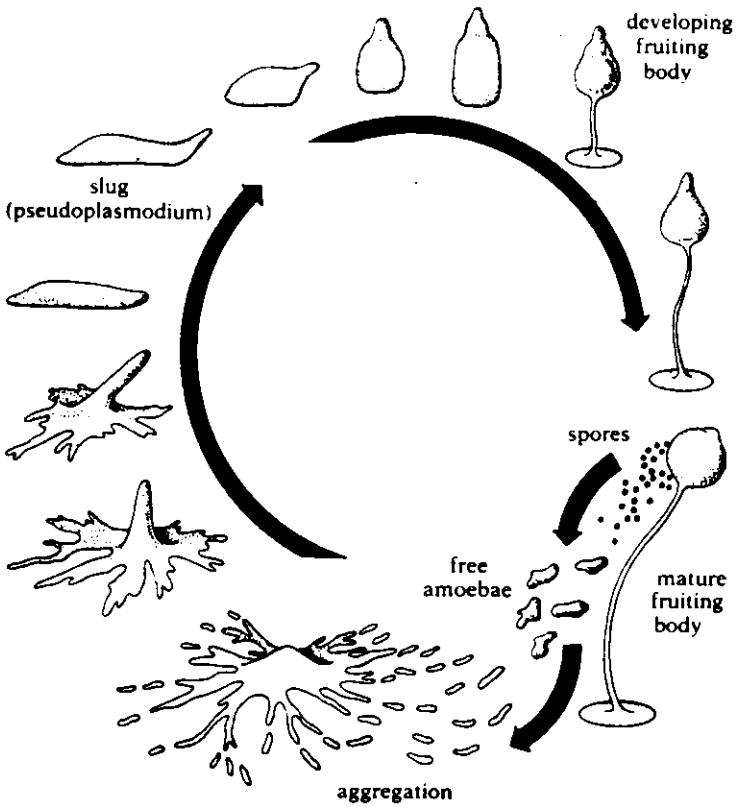


Figure 1. The life cycle of Dictyostelium discoideum

In addition to acting as the chemotactic agent in the development of *D. discoideum*, cAMP has been implicated in the expression and stabilization of many developmentally regulated mRNA species (Sampson et al., 1978; Town and Gross, 1978; Landfear and Lodish, 1980; Barklis and Lodish, 1983; Mangiarotti et al., 1983; Mehdy et al., 1983; Williams et al., 1984; Schaap and Van Driel, 1985). The mechanism or mechanisms by which cAMP-mediated mRNA expression and stabilization occurs is currently the focus of much research. Fluctuations in intracellular cAMP levels are important in regulating many different biochemical processes in other eukaryotes. An intracellular increase in cAMP results from activation of adenylate cyclase, usually in response to extracellular stimuli such as hormones; the physiological effects of elevated intracellular cAMP levels are often the result of a cAMP-dependent protein kinase, an enzyme which is activated by cAMP and which alters the phosphorylation state and activity of substrate enzymes (Walsh et al., 1968). Intracellular cAMP levels can be decreased through the action of phosphodiesterase; decreased cAMP levels inactivate cAMP-dependent protein kinases and may activate phosphatase enzymes which dephosphorylate, and alter the activity of, enzymes which were phosphorylated by the cAMP-dependent kinases. The discovery of a cAMP-dependent protein kinase in *Dictyostelium* (Sampson, 1977; Rutherford et al., 1982, 1984; De Gunzburg and Veron, 1982; Leichtling et al., 1984; Majerfield et al., 1984) raised the possibility that some biochemical processes in this organism are also regulated through a series of cAMP-dependent phosphorylations and dephosphorylations; if such regulation is seen, cAMP acts as both the "first messenger" and the "second messenger". The cAMP-dependent protein kinase may also play a direct role in gene expression; Woffendin et al. (1986) have shown that the kinase enters the nucleus of developing cells of *D. discoideum*, and Chambers et al. (1987) have identified several nuclear proteins as substrates of the enzyme. Recent evidence, however, suggests that cAMP mediated gene expression in *Dictyostelium* occurs directly through the cell surface cAMP receptor in a mechanism independent of the cAMP-dependent protein kinase (Haribabu and Dottin, 1986; Oyama and Blumberg, 1986; Schaap et al., 1986; Mann and Firtel, 1987). Kimmel (1987) has proposed that two distinct mechanisms of cAMP mediated gene expression are seen in *Dictyostelium*.

It should be pointed out that other factors in addition to cAMP have been implicated in gene expression and mRNA stabilization in *Dictyostelium*; for example, cell-cell contact is known to play a role in these these processes (Alton and Lodish, 1977; Chung et al., 1981; Mangiarotti et al., 1981; Blumberg et al., 1982). Also, it has been shown that differentiation inducing factor (DIF), a low molecular weight, hydrophobic compound, can induce pre-stalk specific mRNAs (Williams et al., 1987).

1.3 Glycogen Phosphorylase in Dictyostelium discoideum

A key event in the development of *Dictyostelium discoideum* is glycogen degradation. The degradation of glycogen provides glucose precursors which are used to synthesize components of differentiated cells; one such component is cellulose, which, as mentioned earlier, is a major component of the stalk (Wright et al., 1968; Marshall et al., 1970; Gustafson and Wright, 1972). Glycogen degradation is catalyzed by the enzyme glycogen phosphorylase (1,4-α-D-glucan:orthophosphate α-glucosyl transferase; EC 2.4.1.1). Early research on this enzyme showed it to be developmentally regulated; little or no activity was detected early in development, but activity increased as development progressed and ultimately peaked at culmination (Firtel and Bonner, 1972). Several different studies suggested that the increase in phosphorylase activity during development was due to synthesis of the enzyme molecules. Firtel and Bonner showed that the increase in phosphorylase activity during development would not occur if RNA and protein synthesis were inhibited. Thomas and Wright (1976) found that ^{35}S-methionine incorporation into the phosphorylase protein during development correlated with the increase in enzyme activity, suggesting that the enzyme was being synthesized during development. Higgins and Dahmus (1982) also presented evidence that the enzyme was synthesized during development, and suggested that the developmental regulation of phosphorylase was at the level of mRNA transcription. In light

of the work described above, it was generally accepted that glycogen phosphorylase in *Dictyostelium* was developmentally regulated and accumulated due to *de novo* protein synthesis.

New questions on the developmental regulation of glycogen phosphorylase in *D. discoideum* were raised when Rutherford and Cloutier (1986) identified a previously unknown glycogen phosphorylase activity. Unlike the phosphorylase activity described above, this new form was dependent upon 5′ adenosine monophosphate (5′ AMP) for activity; in this respect it was analogous to the "inactive", or 'b' form of glycogen phosphorylase seen in higher eukaryotes, and was thus called the 'b' form. The glycogen phosphorylase described by Firtel and Bonner (1972), which resembled the mammalian 'a' form of glycogen phosphorylase in that it was the "active" form and was independent of 5′ AMP, was called the 'a' form. Cloutier and Rutherford (1987) showed that the 'b' form of glycogen phosphorylase was present in vegetative amoebae and in the early developmental stages; 'b' activity decreased as development progressed, concomitant with the increase in the activity of the 'a' form. The combined specific activity of the two forms was constant throughout development. In other eukaryotic systems where 'a' and 'b' forms of glycogen phosphorylase have been identified, the inactive 'b' form is converted to the active 'a' form through a cAMP mediated phosphorylation, and the discovery of a 'b' form in *D. discoideum* has raised the possibility that the two forms are developmentally regulated in this manner. Another possiblity is that the two forms represent the products of different genes; if this were the case it would explain the results obtained in the earlier studies of Firtel and Bonner, Thomas and Wright, and Higgins and Dahmus.

In this study, I have examined the relationship between the 'a' and 'b' forms of glycogen phosphorylase in *Dictyostelium* by examining the antigenic similarity of the two forms and by carrying out *in vitro* translation experiments to determine if one or two specific mRNA species exist for the two enzyme forms. Results of this experimentation indicate that the two forms of phosphorylase have different subunit molecular weights and show little antigenic similarity; additionally, results of this experimentation strongly suggest that separate mRNAs do exist for the 'a' and 'b' forms.

2.0 The Relationship Between Two Forms of Glycogen Phosphorylase in *Dictyostelium discoideum*: Antigenic Similarity

2.1 Abstract

The cellular slime mold, *Dictyostelium disoideum*, provides an ideal model system to study eukaryotic cell differentiation. In *D. discoideum*, glycogen degradation provides precursors for the synthesis of developmentally regulated structural products. The enzyme responsible for glycogen degradation, glycogen phosphorylase, exists in active and inactive forms. The active, or 'a' form, is independent of 5'adenosine monophosphate (5'AMP) while the inactive, or 'b' form, is 5'AMP dependent. The activity of the 'b' form predominates early in development while the activity of the 'a' form peaks in mid-late development; their combined specific activities remain constant at any point. Polyclonal antibodies raised to the purified forms of this enzyme show low cross reactivity. The anti-'a' antiserum reacts with a 104 kd protein that is associated with phosphorylase 'a' activity;

the anti-'b' antiserum reacts with a 92 kd protein that is associated with phosphorylase 'b' activity and weakly cross reacts with the 104 kd protein. Cyclic AMP perturbation of intact cells caused induction of both phosphorylase 'a' activity and the 104 kd protein. Immunotitration data suggest that the 'a' form accumulates due to *de novo* protein synthesis, although this result must be interpreted with caution.

2.2 *Introduction*

I am exploring the hypothesis that cAMP (3'5' cyclic adenosine monophosphate) regulates the alternate pathways of differentiation in *Dictyostelium discoideum*. Thus far, the literature has established that cAMP in this slime mold modulates the differentiation of its two cell types, namely spore and stalk cells (Kay et al., 1979). Additionally, the expression and stabilization of various developmentally regulated genes have been attributed to altered intracellular cAMP levels (Sampson et al., 1978; Town and Gross, 1978; Landfear and Lodish, 1980; Barklis and Lodish, 1983; Mangiarotti et al., 1983; Mehdy et al., 1983; Williams et al., 1984; Schaap and Van Driel, 1985). In several systems, a cAMP dependent protein kinase (cAMP Prk) mediates cAMP effects by changing the phosphorylation state of its protein substrates (Walsh et al., 1968). Several labs have recently discovered and characterized a cAMP Prk in *Dictyostelium discoideum* (Sampson, 1977; Rutherford et al., 1982, 1984; De Gunzburg and Veron, 1982; Schoen et al., 1983; Leichtling et al., 1984; Majerfield et al., 1984). As is the case in mammals, a likely substrate for kinase mediated regulation is the enzyme glycogen phosphorylase. This is a realistic hypothesis, since an inactive 5' AMP dependent form (the 'b' form) of the phosphorylase has been discovered (Rutherford and Cloutier, 1986). Previously, only the active, 5'AMP independent form (the 'a' form) of the enzyme was known in this organism. I am now investigating whether the inactive phosphorylase 'b' is activated via a cAMP Prk mediated phosphorylation, or whether the two forms are synthesized as

distinct proteins. Another reason for my interest in glycogen phosphorylase concerns its physiological role in the organism's differentiation. Previous studies showed that this enzyme is active at terminal development, when a cellulose stalk is constructed (Firtel and Bonner, 1972; Thomas and Wright, 1976). Indeed, the glucose units generated by phosphorylase activity are utilized solely for cellulose synthesis, rather than energy metabolism (Wright et al., 1968; Marshall et al., 1970; Gustafson and Wright, 1972). The developmental regulation of the two enzyme forms is thus critical to the development of *D. discoideum*, and I therefore chose to work on the mechanism of developmental regulation of glycogen phosphorylase in this organism. My first step has been the isolation of purified forms of the phosphorylase, and the generation of polyclonal antibodies to each. Presumably the degree of immunological relatedness between these enzyme forms will help determine whether *Dictyostelium* phosphorylase 'a' results from a phosphorylation-mediated conversion of the 'b' form, or whether the two forms of the of phosphorylase represent separate gene products.

2.3 Results

Previous investigations of glycogen phosphorylase in *Dictyostelium* revealed that the enzyme was developmentally regulated. Little or no activity was present in early development, followed by a peak of activity in the culmination stage of development. The activity of this enzyme was not affected by 5'AMP. With the discovery of an inactive 5'AMP dependent form of the phosphorylase (Rutherford and Cloutier, 1986), questions on the relationship between the two enzyme forms and the mechanism of developmental regulation of phosphorylase activity arose. Using DE52 cellulose anion exchange chromatography, Rutherford and Cloutier (1986) demonstrated that the 5'AMP dependent activity (the 'b' form) is present in the early stages of *Dictyostelium* differentiation, namely amoebae and aggregated amoebae. Over the next 18-20 hours,

these multicellular aggregates differentiate into stalk and spore cells, thereby traversing the 'slug' and 'culmination' stages. It is during the latter stages that the 5'AMP independent activity (the 'a' form) increases. Furthermore, the phosphorylase 'b' activity decreases during these later stages. This is observed as a thirteen - fold decrease in the 5' AMP dependence of the enzyme activity (Table 1). Further, Table 1 shows that at any given point in the life cycle, the sum of the specific activities of the 'a' and 'b' forms remains equal, at approximately 250 nmols/min/mg dry wt (Table 1). This value is similar to the maximum *Dictyostelium* phosphorylase activities reported in the literature (Wright et al., 1968; Jones and Wright, 1970; Marshall et al., 1970; Gustafson and Wright, 1972; Firtel and Bonner, 1972; Rutherford and Harris, 1976).

Extracts from each of the stages shown in Table 1 were also subjected to SDS-PAGE, then western blotted, and stained with either anti-'a' or anti-'b' (Figure 2). My aim was to monitor the banding pattern of the phosphorylase protein as its activity changed during development. The phosphorylase from the amoeba stage was entirely in the 'b' form as shown by the 5'AMP dependent activity (Table 1). Upon western blotting of cell extracts from amoebae and rippled amoebae a doublet protein at 92 kd was stained by the anti-'b' antiserum (Fig 2A, lane 1-2). At the aggregation stage a 104 kd protein was weakly stained by the anti-'b' (Fig 2A, lane 3). The presence of this 104 kd protein, as detected by western blotting, coincided with the appearance of 'a' activity as shown in Table 1. At later developmental stages the 104 kd protein split into a doublet, with the upper member of the doublet remaining at 104 kd (Fig 2A, lanes 6,7). The 92 kd protein was also detected in extracts from late developmental stages (Fig 2A, lanes 4-7) and often appeared as a protein doublet. The same cell extracts were also western blotted and stained with the anti-'a' antiserum (Fig 2B). The major protein detected by anti-'a' was the 104 kd protein which appeared at the aggregation stage and persisted throughout the remainder of the developmental cycle; again, a doublet was seen at later stages (Fig 2B, lanes 5-7) with the upper member remaining at 104 kd. As mentioned, the appearance of this 104 kd protein, as detected by both antibodies, coincided with the appearance of 'a' enzyme activity. It is interesting to note that while anti-'b' weakly stained the 104 kd protein the reciprocal was not observed; anti-'a' did not detect the 92 kd protein. It should also be pointed out that the corresponding drop in phosphorylase 'b'

Table 1. Glycogen Phosphorylase Activity During the Life Cycle of *Dictyostelium discoideum*

Stage of Development	Glycogen Phosphorylase Activity (nanomoles/min/mg dry weight)		Total Glycogen Phosphorylase Activity (nanomoles/min/mg dry weight)	Fold Dependence
	"a" form	"b" form		
Amoebae (o.h)	19	236	255	13.5 X
Rippled Amoebae (6 h)	28	189	217	7.8 X
Aggregation (12 h)	141	114	255	1.8 X
Early Slug (14 h)	151	104	255	1.7 X
Migrating Slug (16 h)	132	104	236	1.8 X
Early Culmination (20 h)	245	19	264	non-dependent
Late Culmination (24 h)	217	38	255	non-dependent

0.1 g cells at the desired developmental stage were harvested, lysed, and extracted as described in the experimental procedures. The resulting 14,500 x g supernatants were assayed for glycogen phosphorylase activity as described. Glycogen phosphorylase "a" activity was determined by assaying in the absence of 5' AMP; glycogen phosphorylase "b" activity was determined by assaying in the presence of 5'AMP and subtracting "a" activity from this value.

activity during later developmental stages was not accompanied by diminished intensity of the 92 kd protein (Fig 2A, lanes 4-7). In Figure 2A only one band other than the 92 kd and 104 kd proteins was detected by anti-'b'. This band was a faint lower molecular weight band which may represent a degradation product of the 92 kd protein. In Figure 2B only one band other than the 104 kd protein/doublet was detected by anti-'a' and this was a higher molecular weight band seen in every developmental stage. This band could represent an inactive precursor or an aggregated form of the "a" enzyme, or could be a cross-reactive antigen. Experiments described later lead me to believe that this higher molecular weight protein was probably a cross reacting antigen, since the elution profile of the higher molecular weight protein from anion exchange columns did not match the elution profiles of 'a' activity and the 104 kd protein from the same columns (data not shown).

It has been shown that DE52 cellulose chromatography effectively separated the 'a' and 'b' forms of the phosphorylase (Rutherford and Cloutier, 1986). Using this procedure I obtained fractions containing either the 'a' or 'b' enzymes in order to perform immunoprecipitation and immunoinhibition analyses. Initially, concentrations of antisera and antiserum/protein A ratios were tested in order to optimize conditions for immunoprecipitation. The protocol used (see Experimental Procedures) enabled 90% immunoprecipitation of the antigen(s) from any given extract. Figures 3, 4, and 5 present data from immunoprecipitation analyses of phosphorylase 'a' and 'b' obtained by DE52 chromatography of amoeba and culmination stages. Figure 3 shows western blots of amoeba phosphorylase 'b' activity. These samples were immunoprecipitated then stained with either anti-'b' (Fig 3A) or anti-'a' (Fig 3B). The anti- 'b' stained western blot in Fig 3A shows that the 'b' antiserum immunoprecipitated a protein doublet of 92 kilodaltons (lanes 2,4,6,8). This protein was also weakly immunoprecipitated by the 'a' antiserum (lanes 1,3,5,7). Figure 3B shows the anti-'a' stained blot of the same samples. The only protein observed was the 92 kd protein derived from anti-'b' immunoprecipitations of fractions containing the peak of 'b' form activity (lanes 4,6). The anti-'a' did not immunoprecipitate sufficient enzyme protein from these fractions in order to produce a band detectable by anti-'a' staining (lanes 1,3,5,7).

Figures 4 and 5 show the results of similar experimentation from the culmination stage. At this stage of development DE52 cellulose chromatography clearly separated 'a' and 'b' activity

Figure 2A

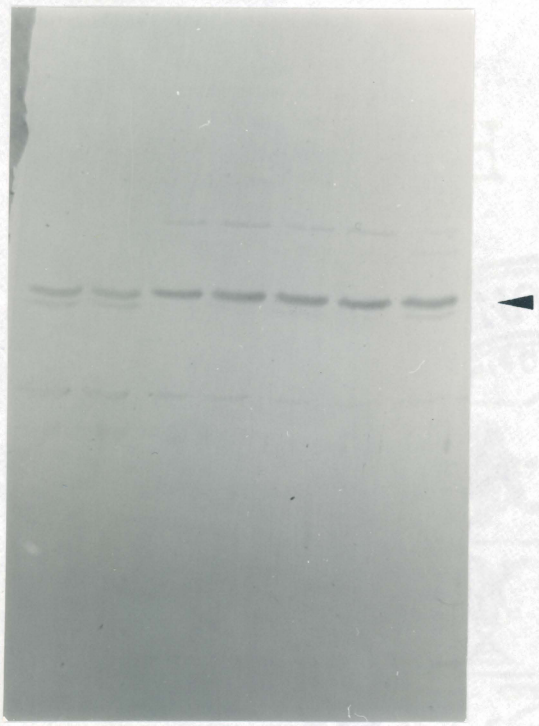


Figure 2B

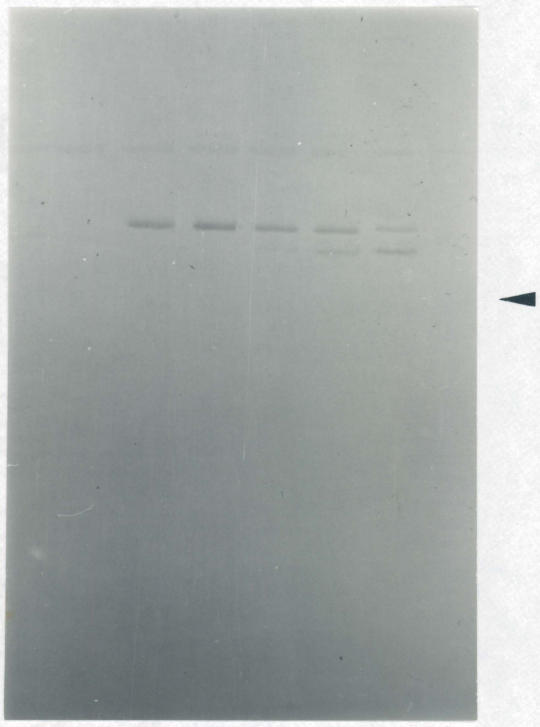
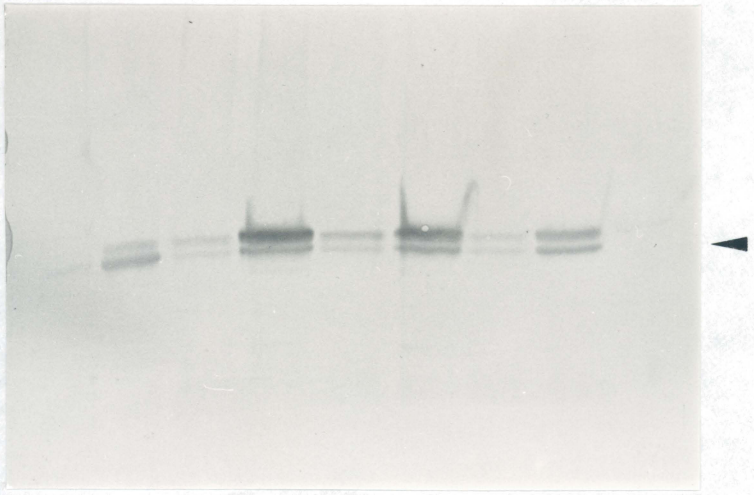


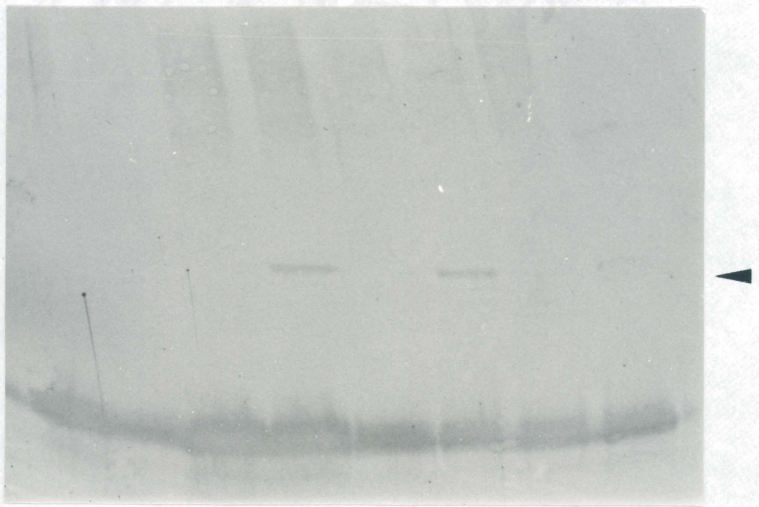
Figure 2. Western blot analysis of phosphorylase 'a' and 'b' activities from seven developmental stages: Cells were harvested in buffer A and lysed by freezing at -20 C. The thawed lysates were spun at 14,500 x g for 6 min. and prepared for SDS-PAGE and western blotting. In both figures: **Lane 1**, amoebae; **Lane 2**, rippled amoebae; **Lane 3**, aggregates; **Lane 4**, early slugs; **Lane 5**, migrating slugs; **Lane 6**, early culmination; **Lane 7**, late culmination. Figure 2A was stained with anti-b while Figure 2B was stained with anti-a. In Figures 2A and 2B the arrow indicates 92kd.

Figure 3A



1 2 3 4 5 6 7 8

Figure 3B



1 2 3 4 5 6 7 8

Figure 3. Immunoprecipitation and staining of amoebae stage phosphorylase with either anti-'a' or *anti-'b'*: Figure 3A: An anti-'b' stained western blot of partially purified 'b' enzyme immunoprecipitated with anti-'b' (Lanes 2,4,6,8) and anti-'a' (Lanes 1,3,5,7). Figure 3B: An anti-'a' stained western blot of partially purified 'b' enzyme immunoprecipitated with anti-'b' (Lanes 2,4,6,8) and anti-'a' (Lanes 1,3,5,7). The arrow indicates 92 kd; 'b' activity was present at peak levels in the fractions which were immunoprecipitated in Lanes 3,4,5, and 6.

peaks (Rutherford and Cloutier, 1986). Thus each form of the enzyme could be analyzed by immunoprecipitation and western blotting. The western blot shown in Figure 4, which was stained with anti-'b', shows that anti-'b' immunoprecipitation of fractions containing 'b' activity yielded the 92 kd protein (lanes 2,4,6,8,10). The 'a' antiserum weakly immunoprecipitated this protein from the same samples (lanes 1,3,5,7,9), suggesting low cross-reactivity between antisera.

Similar experiments on DE52 fractions containing only 'a' activity revealed an interesting difference in that the 92 kd protein was absent and was replaced by a protein doublet of 104 kd (Fig 5A,5B). The 104 kd doublet was immunoprecipitated by the 'a' antiserum only (Fig 5A,5B, lanes 1,3). The intensity of the 104 kd protein (as measured by immunostaining) paralleled the phosphorylase 'a' activity in these DE52 cellulose fractions (as measured spectrophotometrically). Although the anti-'b' antibody could not immunoprecipitate the 104 kd protein (Fig 5A, 5B), the anti -'b' could recognize the 104 kd protein band since this protein was stained on nitrocellulose (Fig 5A). This result may be due to the greater affinity of anti- 'b' for denatured phosphorylase 'a' on nitrocellulose as opposed to the native enzyme that was present in fractions used in the immunoprecipitations.

Careful analysis of the phosphorylase activity in the individual DE52 fractions and the intensity of the immunostaining of these same fractions after western blotting revealed indentical patterns for the elution of the 'b' activity - 92 kd band, and for the 'a' activity - 104 kd band. These results are in agreement with the activity and banding pattern of the phosphorylase as shown in Figure 2.

The specificity of the antisera for their respective forms of phosphorylase was also tested by immunoinhibition of enzyme activity from the DE52 fractions containing either 'a' or 'b' activity. Table 2 shows that the 'b' antiserum did not inhibit phosphorylase 'a' activity, whereas the 'a' antiserum caused a 67% inhibition of 'a' activity. Conversely, phosphorylase 'b' activity was completely inhibited by the anti-'b' antibody, and marginally inhibited by anti-'a' antiserum.

The data presented in Figures 2 through 5 suggest that in partially purified extracts the phosphorylase 'b' monomer has a molecular weight of 92 kd, whereas the phosphorylase 'a' monomer corresponds to the 104 kd protein. In order to test this result further, I subjected the

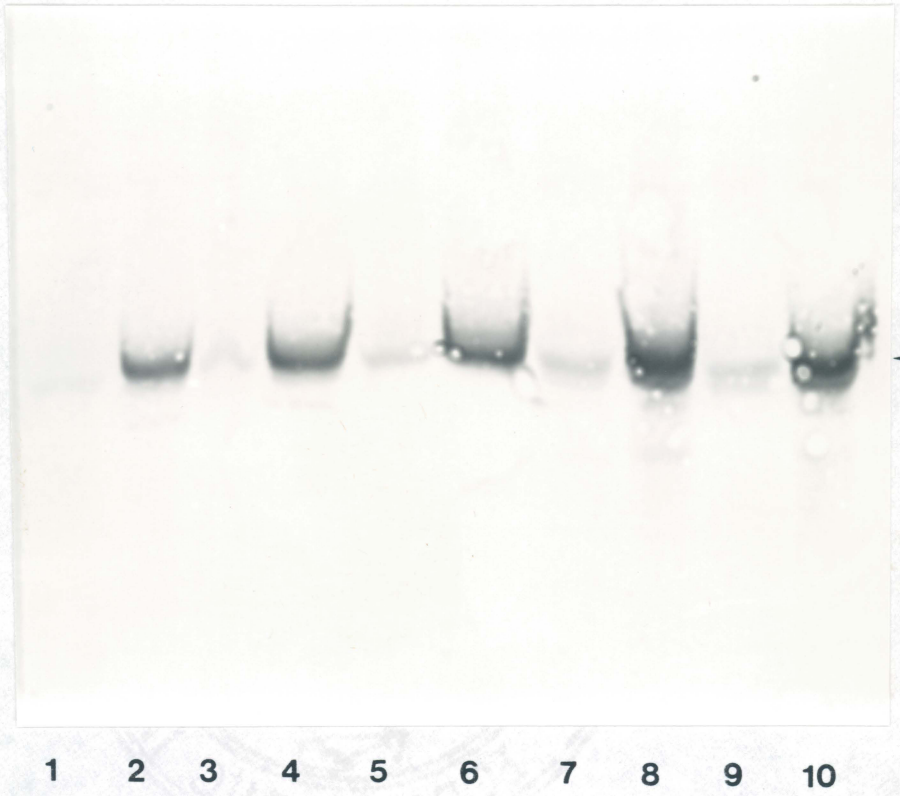


Figure 4. Culmination stage phosphorylase 'b' activity from DE52 cellulose chromatography immunoprecipitated with both antisera: In Lanes 1,3,5,7,9 the phosphorylase 'b' activity peak was immunoprecipitated with anti-'a'. In Lanes 2,4,6,8,10 the 'b' activity peak was immunoprecipitated with anti-'b'. The arrow indicates 92 kd. Phosphorylase 'b' activity was present in increasing levels moving from Lane 1 to Lane 10.

Figure 5A

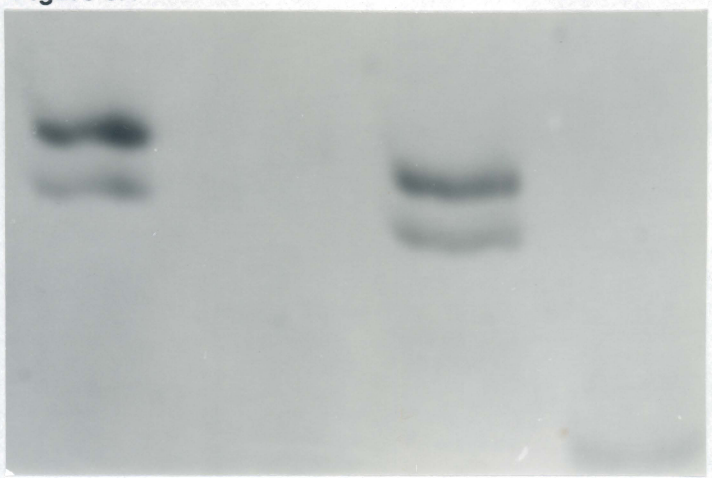


Figure 5B

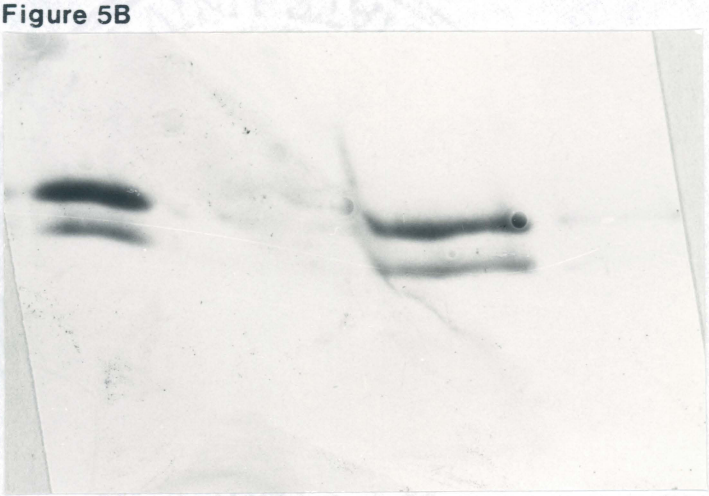


Figure 5. Culmination stage phosphorylase 'a' activity immunoprecipitated, western blotted, and stained with both antisera: Figure 5A: An anti-'b' stained blot of phosphorylase 'a' activity immunoprecipitated with anti-'a' (Lanes 1,3) and anti-'b' (Lanes 2,4). Figure 5B: An anti-'a' stained blot of phosphorylase 'a' activity immunoprecipitated with anti-'a' (Lanes 1,3) and anti-'b' (Lanes 2,4). The arrows indicate 92kd.

Table 2. Immunoinhibition Analyses of Glycogen Phosphorylase 'a' and 'b' Activities

Antiserum used	Percent Inhibition of "a" activity	Percent Inhibition of "b" activity
anti-a	67%	15%
anti-b	0%	97%

Glycogen phosphorylase "a" and "b" activities from slug stage cell extracts were eluted from a DE52 column and were adjusted to 7.5 nmol/ml/min. Antisera (diluted 1:5 in Buffer A) were incubated with equal volumes of enzyme overnight at 4°C and then centrifuged at 12,000 x g for 10 min; activity remaining in the resultant supernatant was assayed and percent inhibition was calculated. Preimmune controls were performed in all cases; no inhibition was seen with preimmune serum.

DE52 cellulose fractions containing 'a' activity to an additional purification by hydroxylapatite chromatography; an additional seven-fold purification of phosphorylase was obtained using this procedure. Fractions eluted from the hydroxylapatite column which contained 'a' activity were subjected to SDS-PAGE and western blotting. Figure 6 shows that the hydroxylapatite purified phosphorylase 'a' protein comigrated with the 104 kd protein and is therefore clearly of higher molecular weight than the 'b' form. The blot shown in Figure 6 was stained with a mixture of both antisera in order to visualize both the 92 kd and 104 kd proteins. Again the pattern of elution from the column was identical for the phosphorylase activity and for the 104 kd protein.

Cyclic AMP (cAMP) perturbation of intact amoebae and the subsequent analysis of the glycogen phosphorylase lends further support to the hypothesis that the 104 kd protein represents *Dictyostelium* phosphorylase 'a'. The literature has shown that exogenously added cAMP induces phosphorylase activity in shaken cell suspensions wherein cell contact is negligible (Schaap and Van Driel, 1985). Figure 7 shows parallel induction by cAMP of 5'AMP independent phosphorylase activity and the appearance, upon western blotting, of the 104 kd protein. The induction of phosphorylase 'a' activity by cAMP was accompanied by a corresponding decrease in phosphorylase 'b' activity. Typically, a 70-90% decrease in 'b' enzyme activity was accompanied by a corresponding 70%-90% increase in the 'a' enzyme activity (Figure 8).

The cAMP induction of the phosphorylase 'a' could be due to synthesis of the enzyme protein, or to cAMP activation of a cAMP Prk and eventual phosphorylation of the glycogen phosphorylase. In order to determine if the cAMP-induced 'a' protein was due to phosphorylation, the cAMP induction experiments were repeated in medium containing ^{32}P- orthophosphate. In these experiments, a phosphorylated 92 kd protein was observed both in the presence and absence of cAMP. The 104 kd protein did not appear to be phosphorylated in the flasks containing cAMP although induction of the 'a' activity occurred. These data do not necessarily rule out cAMP dependent phosphorylation as the mechanism of phosphorylase activation for the following reason: no cAMP dependent phosphorylation of proteins was detected in these experiments (as measured by autoradiography) although cAMP Prk activity was measured in flasks shaken in the presence or absence of cAMP. The kinase was 6-8 fold dependent upon cAMP in both types of flasks,

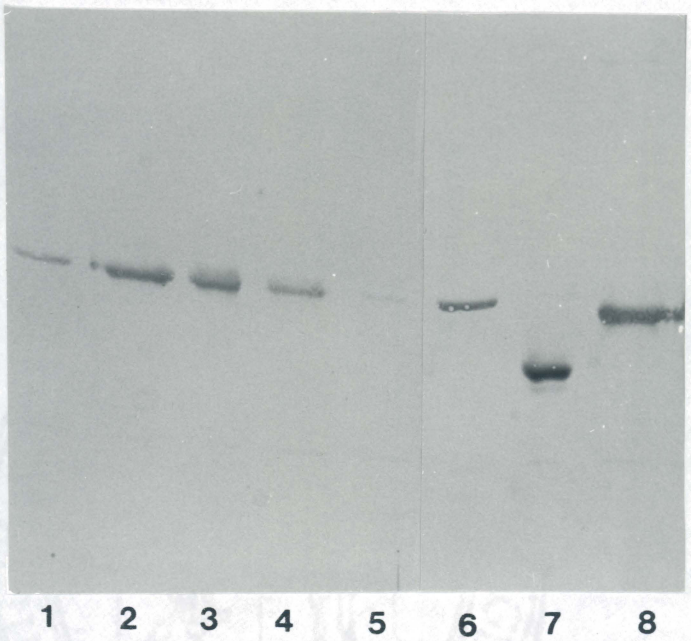
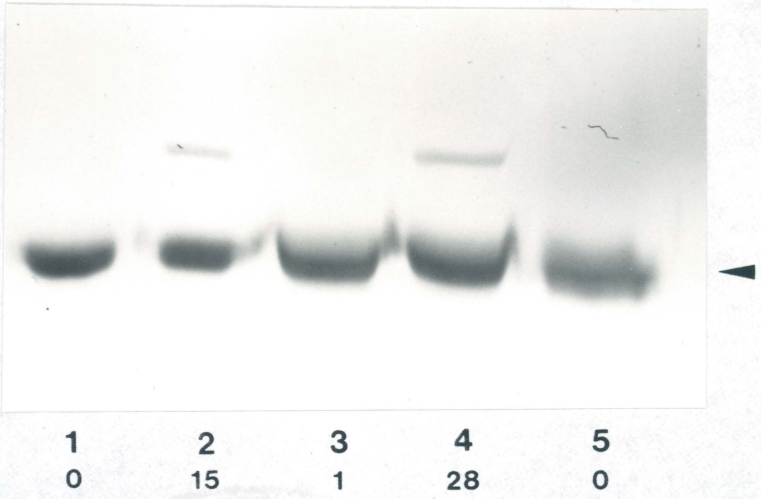


Figure 6. Molecular weight of hydroxylapatite purified phosphorylase 'a': Phosphorylase 'a' from culmination stage cells was purified to near homogeneity by DE52 anion exchange and hydroxylapatite chromatographies. The resulting protein was subjected to SDS-PAGE, western blotted, and stained with a mixture of anti-'a' and anti-'b'. **Lanes 1-5,** hydroxylapatite column fractions containing varying levels of phosphorylase 'a' activity; **Lanes 6-7,** the 104 kd and 92 kd proteins, respectively, from slug stage cells; **Lane 8,** hydroxylapatite purified phosphorylase 'a'.

Figure 7A



1	2	3	4	5
0	15	1	28	0

Figure 7B

1	2	3	4	5
0	15	1	28	0

Figure 7. cAMP induction of phosphorylase 'a' activity in shaking cultures: Aggregation competent amoebae were suspended at a density of 5 x 10^6 cells/ml in Buffer D in the presence or absence of 1 mM cAMP. After 4 and 8 hours of shaking, 5 ml of the cell suspension were removed, sonicated, and assayed for phosphorylase activity. A portion of each sample was western blotted and stained with either anti-'b' antiserum (Fig 7A) or with anti-'a' (Fig 7B). In both figures: **Lane 1**, 4 hours shaking without cAMP; **Lane 2**, 4 hours shaking with 1 mM cAMP; **Lane 3**, 8 hours shaking without cAMP; **Lane 4**, 8 hours shaking with 1 mM cAMP; **Lane 5**, aggregation competent cells. The numbers below each lane indicate glycogen phosphorylase 'a' activity (nmole/min/mg wet wt). The arrows indicate 92 kd.

suggesting that it was present as a holoenzyme. However, the actual state of dissociation of the catalytic and regulatory subuntits of the kinase *in vivo* was not measurable, since it has been shown previously that the subunits have rapid reassociation kinetics (Vaughan, 1985). Therefore, a true estimate of cAMP Prk activity and that of its putative substrate phosphorylase kinase cannot be determined under these experimental conditions. Experiments are currently in progress to determine whether the cell surface cAMP receptor is involved in mediating cAMP's inductive effect on phosphorylase 'a'. The literature suggests that this receptor activates gene expression via a mechanism that does not involve the cAMP Prk (Haribabu and Dottin, 1986; Oyama and Blumberg, 1986; Schaap et al., 1986; Mann and Firtel, 1987).

One method that is commonly used to examine whether an increase in enzyme activity is due to accumulation of enzyme molecules (*de novo* synthesis) or due to an activation of preexisting enzyme is immunotitration (Mayer and Walker, 1980). Immunotitrations were carried out on dilutions of 14,500 x g supernatants from different developmental stages. The results of immunotitration with the anti-'a' antibody are shown in Figure 9. The equivalence points (the concentration of 'a' antiserum required to completely inhibit phosphorylase 'a' activity from a given extract) for all three stages were identical. This would normally indicate that the enzyme activity/molecule of enzyme protein is the same for all stages of development. Therefore, the increase in activity of the 'a' form during development would be attributed to an increase in the number of enzyme molecules, rather than to activation of a preexisting inactive form. Although immunotitration is a commonly used technique for distinguishing between *de novo* synthesis and activation of inactive enzyme, there are some considerations that should be evaluated in this particular case. For example, implicit in the experiment is that antibody against an active form of the enzyme will cross-react with the inactive form. If this were true, then competition between the active and inactive forms would occur during immunotitration, resulting in different equivalence points as phosphorylase 'b' to 'a' conversion occurs. However, if the antibody does not cross-react well with the inactive form, then identical equivalence points would result--even though the mechanism by which the active form appears is via activation of the inactive form. I therefore interpret the results in Figure 9 with caution, for if the actual mechanism of the appearance of the 'a' activity

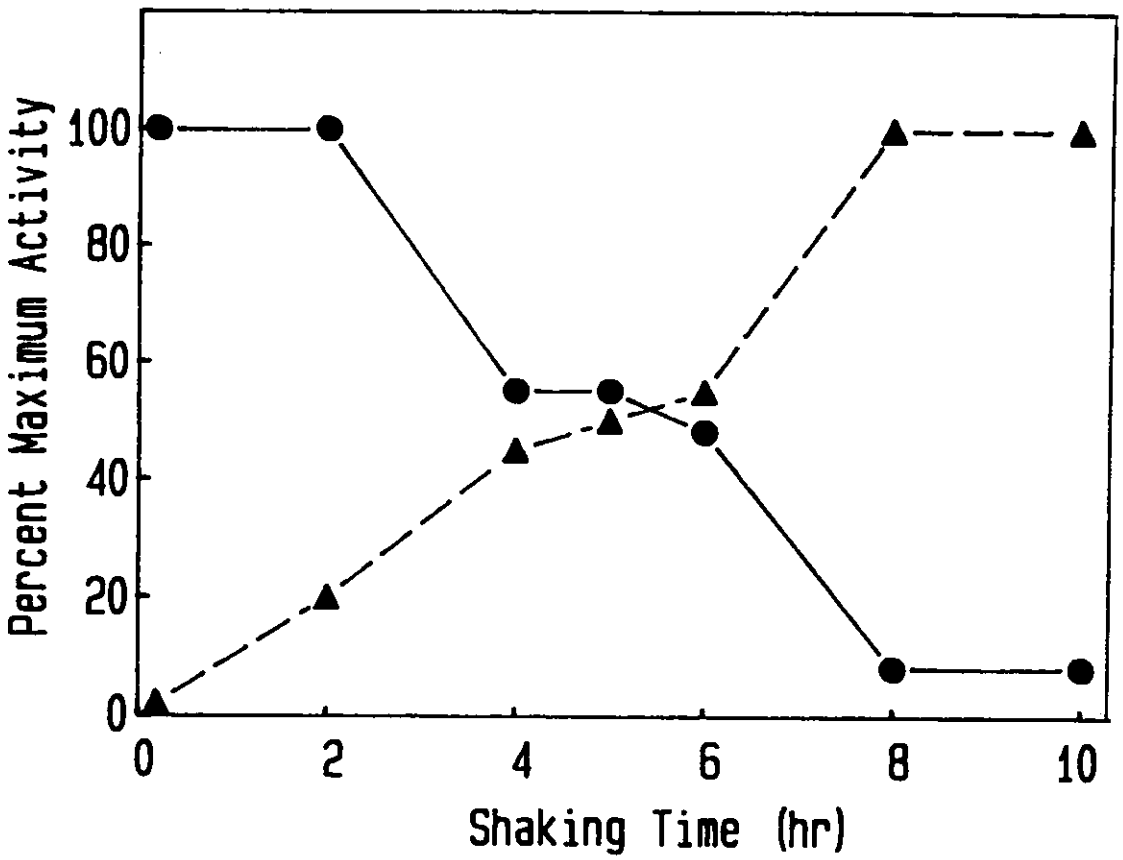


Figure 8. **Glycogen phosphorylase activity in cells shaken in the presence of cAMP:** Aggregation competent cells were suspended at a density of 5 x 10^6 cells/ml in buffer D containing 1 mM cAMP. Phosporylase activity was assayed at the indicated time points. Phosphorylase 'a' activity is indicated by the closed triangles (▲) and was assayed in the absence of 5'AMP. Phosphorylase 'b' activity is indicated by the closed circles (●) and was assayed in the presence of 5'AMP.

is by conversion of the 'b' form nearly identical equivalence points would be expected since we have shown that the anti-'a' cross-reacts poorly with the 'b' form. Alternately, if the anti-'a' does not cross-react with the 'b' form because it represents a separate protein, the immunotitration as shown in Figure 8 is a valid indication that the 'a' form appears as the result of *de novo* synthesis. This argument hinges on the low cross-reactivity between anti-'a' and the 'b' enzyme. If phosphorylation were the mechanism of phosphorylase 'b' to 'a' conversion, it is conceivable that the resulting conformational change would create significant antigenic differences between the two enzymes forms, and therefore result in the low cross-reactivity between antisera (Table 2).

2.4 Discussion

The immunological data presented above show that the anti- 'a' antibody reacts with a 104 kd doublet protein, and cross-reacts weakly with a 92 kd protein. The 'b' antiserum on the other hand reacts with the 92 kd protein and cross-reacts weakly with the 104 kd protein. The low crossreactivity is demonstrated in Table 2, where only 0-15% percent crossreactivity exists between antisera. Since the low crossreactivity was demonstrated by two different techniques, immunoprecipitation and immunoinhibition, I suggest that these data reflect molecular differences between the two forms of the enzyme. Whether these molecular differences arise due to altered conformation of the 'a' protein due to conversion of the 'b' form via phosphorylation, or other covalent modifications, or is due to the action of different genes cannot be determined by these results alone. Unless some epigenetic mechanism of activation of the 'b' form is involved, it is difficult to explain the concomitant decrease of 'b' activity and increase of 'a' activity with a constant combined specific activity during development. It is interesting to note that most lower eukaryotes that exhibit two forms of glycogen phosphorylase utilize a phosphorylation mediated conversion mechanism (Tellez-Inon and Torres, 1970; Fosset et al., 1971; Hayakawa, 1985).

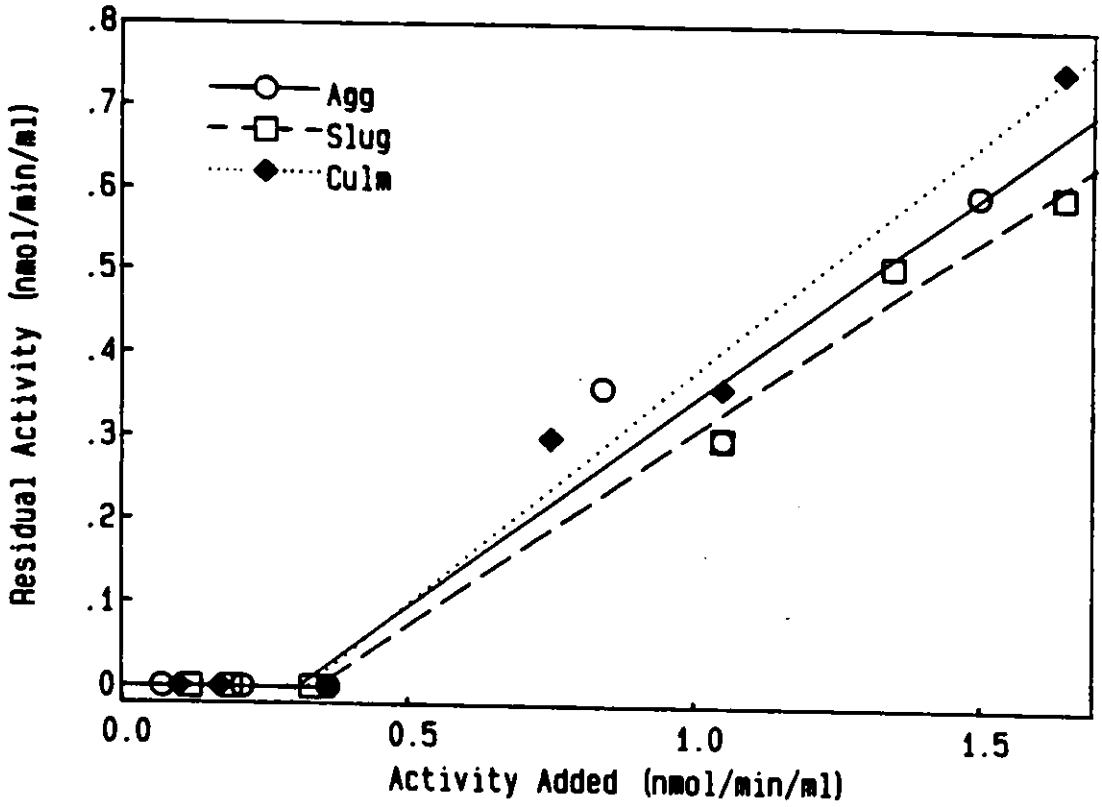


Figure 9. Immunotitration of phosphorylase 'a' activity from crude extracts by anti-'a' antiserum: Cells at the aggregation, slug, and culmination stages were lysed by sonication and spun at 14,500 x g for 10 min. The resulting supernatants were diluted in Buffer A in order to obtain 15 nmoles/min/ml of 'a' activity. A 0.2 ml aliquot of each diluted sample was incubated with anti-'a' antiserum and protein A as described. The residual activity vs. initial phsophrylase 'a' activity was plotted for each stage.

These data also strongly suggest that the 104 kd protein represents *Dictyostelium* phosphorylase 'a'. The appearance of the 104 kd protein parallels the appearance of 'a' enzyme activity during development (Fig 2, Table 1). This protein is specifically detected on western blots by the anti - 'a' antibody from crude extracts of cAMP induced cells (Fig 7), and is specifically immunoprecipitated from partially purified phosphorylase 'a' samples (Fig 5). The significance of the doublet versus singlet form of the the 104 kd protein remains unclear at this point. On the other hand, the phosphorylase 'b' activity is clearly associated with the 92 kd protein (Fig 3, Fig 4). The 92 kd protein can be specifically immunoprecipitated by anti-'b' from partially purified phosphorylase 'b' preparations from either the amoeba or culmination stages.

Previous reports had shown that *Dictyostelium* phosphorylase 'a' is a 5'AMP independent enzyme that appears in mid-late development (Jones and Wright, 1970; Marshall et al., 1970; Firtel and Bonner, 1972; Thomas and Wright, 1976). The data on the molecular weight of this enzyme appear ambiguous. Thomas and Wright (1976) reported a molecular weight of 210 kd for partially purified phosphorylase 'a' by gel filtration analysis. They also reported a molecular weight of 200 kd for the purified enzyme by nondenaturing gel electrophoresis. However, the subunit molecular weight of purified phosphorylase 'a' observed by them was 95 kd, rather than an expected figure of 100-105 kd, based on their gel filtration and nondenaturing gel electrophoresis data. Further, Thomas and Wright (1976) were able to immunoprecipitate a 95 kd protein with antibody raised to a purified phosphorylase 'a' preparation. Higgins and Dahmus (1982), however, observed 101 and 105 kd proteins that crossreacted with the antibody against their 90 kd phosphorylase 'a' subunit. Furthermore, they obtained synthesis of a 105 kd phosphorylase 'a' subunit by *in vitro* translation of *Dictyostelium* mRNAs in the presence of 1 mM PMSF. They concluded that the 105 kd protein represented an inactive precursor of the 'a' enzyme. It is possible that the 90-95 kd protein that Thomas & Wright and Higgins & Dahmus reported as the subunit of phosphorylase 'a' may have been the 'b' enzyme subunit. The 'b' subunit may have cross-reacted strongly with their anti-'a' antibody. However, this would not explain why these earlier reports suggested that the 90-95 kd protein was synthesized late in development when 'b' activity is decreasing. Alternately, the 90-95 kd protein observed by these investigators may have represented proteolysed or

dephosphorylated active 'a' enzyme. If this possibility is applied to the 92 kd protein described in this report it would not explain why the anti-'a' antibody cross-reacted weakly with the 92 kd protein, since both the anti-'a' and anti-'b' strongly recognized degradation products of their respective antigens (data not shown). Additionally, if our 92 kd protein represented a truncated but active 'a' enzyme, it would be difficult to explain why this protein has always been associated with 5'AMP dependent 'b' activity that peaks in early development (Figs 2,3,4, Table 1).

Thomas and Wright (1976) concluded that the increase in 'a' enzyme activity during development was due to *de novo* protein synthesis, since the specific radioactivity of immunoprecipitated enzyme corresponded with the amount of acid insoluble protein obtained at each developmental stage. They were unable to perform this experiment with amoebae and aggregation stages due to the low activity of the enzyme at these stages. This, together with the fact that their antibody may have cross- reacted strongly with the phosphorylase 'b', may have prevented them from detecting a potentially unstable 104 kd 'a' protein immunologically.

The apparent 104 kd molecular weight of the 'a' form of the enzyme does not necessarily mean that the 'a' form is a larger protein and therefore represents a distinct gene product, for evidence is present in the literature suggesting that phosphoproteins may show retarded mobility in SDS gels and thus show an apparent molecular weight increase (Dahmus, 1981). Naranan et al. (1988, manuscript submitted) demonstrated that the 92 kd protein could be phosphorylated *in vitro* but this phosphorylation did not alter the apparent molecular weight of the protein; however, this phosphorylation did appear to convert the enzyme activity from 5' AMP dependent to 5' AMP independent. Similar experimentation did not result in phosphorylation of the 104 kd protein (Naranan, personal communication).

In conclusion, glycogen phosphorylase 'a' in *Dictyostelium discoideum* shows an apparent subunit molecular weight ot 104 kd while glycogen phosphorlyase 'b' shows an apparent subunit molecular weight of 92 kd. This apparent molecular weight difference may indicate that the two forms of phosphorylase are distinct proteins, and immunotitration data, although interpreted with caution, also support this possibility. However, these data alone are not conclusive. I therefore

turned to examining when the mRNA(s) for the two enzyme forms are present throughout development; this experimentation is described in Chapter 3.

2.5 Materials and Methods

Materials

[γ^{32}P] ATP (25 Ci/mmol) was purchased from ICN. Nitrocellulose was purchased from Fisher Products, DE52 cellulose from Whatman, and Protein A (Pansorbin cells) from Calbiochem. Other reageants were purchased from Sigma Chemicals.

Cell Harvest and Development.

Dictyostelium discoideum (AX-3) was grown in liquid HL5 media on a rotary shaker as previously described (Rutherford and Cloutier, 1986). The resulting amoebae were harvested by centrifugation in a continuous flow rotor, then were washed and resuspended in 50 mM Tris-HCl, pH 7.5, containing 2 mM benzamidine, 2 mM mercaptoethanol and 0.02% sodium azide (buffer A). When differentiated cells were required, the amoebae were washed free of media, diluted 1:3 (weight:volume) in 7 mM N-morpholino ethanesulfonic acid, pH 6.5, containing 20 mM KCl and 5 mM $MgSO_4$ (buffer B) and plated on non-nutrient agar. For DE52 chromatography of amoeba and culmination stages, approximately 25 grams of cells from each stage were harvested in 5 volumes of cold buffer A. All subsequent steps were carried out at 4°C.

The stage study in Fig 2 was carried out as follows. Amoebae were pelleted by centrifugation at 1,800 x g for 4 min, and were washed once in buffer B. These cells were resuspended in thrice the volume of buffer, then 0.4 ml of this suspension were plated onto Gelman GN-6 membrane filters supported by Gelman absorbent pads. Both the membranes and pads were pre-soaked for 30 min in buffer B. At the required stage, the membranes were frozen at -70°C. Upon thawing, 0.5 ml of buffer A was applied to each pad. The lysates were scraped from the membranes and then centrifuged at 14,500 x g for 6 min. The supernatant was used to prepare gel samples for SDS- Polyacrylamide gel electrophoresis (SDS-PAGE) as described below.

Preparation of Cell lysates

Washed cells were suspended in 5 volumes of cold buffer A and sonicated by subjection to three 45 second exposures to a 2 cm probe of a sonic cell disrupter (Model 300, Fisher). This lysate was centrifuged for 20 mins at 8000 x g and the resulting supernatant centrifuged at 100,000 x g for an hour. Differentiated cells were homogenized with three strokes of a Potter-Elvehjem tissue grinder and then sonicated as described for amoebae.

Column chromatography

The 100,000 x g supernatant was batch treated with 0.1 volume of DE52 cellulose resin that had been equilibrated in buffer A. After stirring 10 min, the resin was settled by centrifugation. Bound proteins were eluted with an 8 hour linear 0 - 0.25 M KCl gradient in buffer A. Phosphorylase 'a' activity eluted at 50 mM KCl, while the 'b' form eluted at 180 mM KCl. Column fractions were then assayed for glycogen phosphorylase activity in the presence and absence of 5' AMP as described below. Hydroxylapatite chromatography was run on pooled and ten-fold

concentrated DE fractions containing "a" activity. Bound proteins were eluted using a 4h linear 50 mM - 100 mM potassium phosphate (pH 7.0) gradient.

Enzyme Assay

A 20 μl sample of a given enzyme sample was added to 200 μl of reaction mixture containing 50 mM Imidazole (pH 6.8), 2.5 mg/ml glycogen, 5 mM $MgCl_2$, 0.5 mM NADP, 50 μg/ml glucose-1-6 diphosphate, 2 mM K_2HPO_4, 0.3 μmol/ml glucose-6-phosphate dehydrogenase, and 0.4 μmol/ml phosphoglucomutase. The temperature of the assay mixture was maintained at approximately 23°C. A molar extinction coefficient of 6.2×10^3 was used to quantitate NADPH formation at 340 nm. One unit of activity is defined as the amount of enzyme that catalyzes the synthesis of 1 μmol NADPH / min at 23 °C.

Immunoprecipitation analyses

Phosphorylase 'a' and 'b' activity peaks obtained by DE52 Cellulose chromatography were used for these experiments. Typically 0.5 ml of an active fraction with activity ranging from 5-20 nmoles/min/ml were incubated with 0.1 ml of crude, undiluted antiserum for 45 mins at 25 °C. Protein A (20 mg) was then added in order to pellet the antigen - antibody complex (1800 x g for 8 mins). The resulting pellet was washed five times in order to remove proteins bound nonspecifically to the protein A (wash 1, 2 ml of 0.1% SDS in H_2O; wash 2, 2 ml of 0.1% SDS; washes 3-5, 3 ml each of cold buffer A). The immune complexes were eluted from the protein A pellet by boiling for 2 min in 0.1 ml buffer A containing 2% SDS and 5% dithiothreitol. After pelleting the protein A, the supernatant was mixed with sucrose crystals and 0.02% pyronin Y in preparation for SDS-PAGE.

Immunoinhibition analyses

Phosphorylase 'a' and 'b' activities from DE52 chromatography were used for these analyses. The respective activities were equalized to approximately 15 nmoles/min/ml by dilution in buffer A. A 0.1 ml sample of each antiserum (1:5 in buffer A) was incubated with 0.1 ml of the enzyme fraction overnight at 4°C and then centrifuged at 12,000 x g for 10 mins. The resulting supernatants were then assayed for phosphorylase activity as described above. Percent inhibition of enzyme activity was calculated by comparing the activities in samples incubated with antisera versus those incubated with a preimmune control serum.

Immunotitration

Crude extracts from the aggregation, slug and culmination stages were prepared as described earlier for the stage study. The phosphorylase 'a' activity from each of these stages was adjusted to 15 nmoles/min/ml by making appropriate dilutions in buffer A. A 0.2 ml sample of each of these dilutions was incubated with 25 μl of anti -'a' for 45 mins at room temperature. After a 25 min incubation with 2.5 mg of protein A, the antigen - antibody complex was pelleted as described above. The resulting supernatants were assayed for residual phosphorylase 'a' activity. Fig 8 was generated by plotting residual 'a' activity versus initial phosphorylase 'a' activity for each stage.

Western Blotting

Proteins resolved by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes using a Hoefer transfer chamber and a power source (model TE51) set at 1.2 amps for one hour. The transfer buffer contained 192 mM glycine and 20% methanol in 25 mM Tris-HCl,

pH 8.3. After transfer, the nitrocellulose was placed in 50 ml of 50 mM Tris-HCl, pH 7.6, 200 mM NaCl and 0.1% Tween 20 (buffer C), and gently shaken for 20-30 min. The buffer was then replaced with 50 ml of fresh buffer C containing 100 μl - 300 μl of the undiluted antiserum. After 1-12 hours of incubation with the antibody, the nitrocellulose was rinsed several times with fresh 50 ml solutions of buffer C. A 50 ml solution of buffer C containing 1 μg/ml protein A - peroxidase was then placed onto the blot and incubated with gentle shaking for 1hr. The nitrocellulose was rinsed with two 50 ml volumes of buffer C and then with 50 ml of buffer C lacking Tween 20 (buffer C'). The nitrocellulose was then exposed to 48 mls of peroxidase reaction mixture containing 17% methanol, 24 mg of 4-chloro-1-napthol, and 0.008% H_2O_2 in buffer C for 30-120 min. In some cases, the blots were also stained for protein using 0.1% napthol-blue black (amido black) in methanol, water, and acetic acid (4.5:4.5:1 ratio, v/v/v). The destaining solution contained 90% methanol and 2% acetic acid. Autoradiography of the blots was performed using a Kodak intensifying screen and Kodak XAR-5 X-ray film.

Antibody Preparation

Both the 'a' and 'b' forms of the phosphorylase were purified to homogeneity as described previously (Rutherford and Cloutier, 1986; Cloutier and Rutherford, 1987). These proteins were subjected to preparative SDS-PAGE, and were then eluted from the gel slices electrophoretically. The eluates were used as antigens. Two New Zealand white rabbits were each injected with 50-150 μg of purified protein in complete Freunds adjuvant. The rabbits were reinjected with 50-100 μg protein in incomplete Freunds adjuvant at two week intervals. After 6 weeks, the presence of antibody to the protein was confirmed by western blotting. Whole blood was allowed to clot overnight at 4 °C, then centrifuged at 10,000 x g for 10 min. The serum was divided into 300 μl aliquots and stored frozen at -20 °C for subsequent use. Both antiserum preparations had similar titres; a 1:500 dilution of each serum was used for immunostaining and immunoprecipitation.

cAMP Induction experiments

Logarithmically growing AX-3 amoebae were plated onto non-nutrient agar in buffer B and then placed at 4°C for 12 hrs. One aliquot of these starved cells was removed from the agar, washed twice in buffer B, and resuspended at 5 x 10^6 cells/ml in buffer B containing 1mM Na-K phosphate, pH 7.0 (Buffer D), either in the presence or absence of 1 mM cAMP. The cells were placed in 125 ml Erlenmyer flasks (30 ml per flask) and were shaken at 150 rpm on an orbital shaker (Model 3520, Lab-Line Instruments). At 0, 4, and 8 hours post shaking, 5 ml of cells were removed from each flask. These cells were centrifuged, (1,800 x g for 4 minutes), resuspended in Buffer A, and lysed by sonication as explained earlier. The lysates were centrifuged at 14,500 x g for 6 mins. The resulting supernatants were assayed for phosphorylase activity, and prepared for SDS-PAGE samples immediately.

3.0 The Relationship Between Two Forms of Glycogen Phosphorylase in *Dictyostelium discoideum*: In Vitro Translation Analyses

3.1 Abstract

In the slime mold *Dictyostelium discoideum* glycogen phosphorylase exists in active and inactive forms. The inactive, or 'b' form, requires 5' AMP as a positive allosteric modifier for activity, while the activity of the active, or 'a' form, is independent of 5' AMP. The two forms are developmentally regulated and show different subunit molecular weights. The 'a' activity increases in the late stages of the developmental cycle of *Dictyostelium* and is associated with a 104 kd protein; 'b' activity decreases during development and is associated with a 92 kd protein. In this report I present *in vitro* translation studies that suggest that there are two separate mRNA species for the two enzyme forms. The mRNA for the 'b' form appears to be present throughout development while the mRNA for the 'a' form appears to be present only in later developmental stages.

3.2 Introduction

Glycogen phosphorylase plays a major role in the developmental cycle of *Dictyostelium discoideum*. This enzyme catalyzes the breakdown of glycogen, and the resultant glucose units are used to synthesize components of differentiated cells (Wright et al., 1968; Gustafson and Wright, 1972). Two developmentally regulated forms of glycogen phosphorylase have been identified in *D. discoideum* (Rutherford and Cloutier, 1986). The 'b' form is inactive in the absence of 5' AMP, which serves as a positive allosteric modifier; the 'a' form is independent of 5'AMP. The two forms are developmentally regulated. Early in development, the activity of the 'b' form predominates. As development progresses, the 'a' activity becomes detectable and ultimately, by the culmination stage of development, is the predominant form; 'b' activity shows a concomitant decrease during development as 'a' activity increases (Cloutier and Rutherford, 1987). The developmental regulation of these two enzyme forms results in the developmentally regulated degradation of glycogen, since the 'a' form is believed to catalyze glycogen breakdown *in vivo* (Cloutier and Rutherford, 1987); thus, the regulation of the 'a' and 'b' forms plays a very important role in this organism's development. Two possible mechanisms of regulation exist. First, the inactive 'b' form could be converted to the active 'a' form through a covalent modification, such as phosphorylation, as is seen in other eukaryotic systems. Second, the two forms could be synthesized as distinct proteins. This second possibility also has some precedence in the literature, as it has been shown that fetal and adult isozymes of phosphorylase in other organsims are distinct gene products (Berndt et al., 1987). I have shown that the 'a' form has an apparent subunit molecular weight of 104 kd, while the 'b' form has an apparent subunit molecular weight of 92 kd. This molecular weight difference, however, does not conclusively prove that the two forms are distinct proteins and the products of separate genes since phosphorylation of a protein can alter that protein's mobility during SDS-PAGE (Dahmus, 1981). To gain further insight into the relationship between the two forms of phosphorylase, I have attempted to determine if one or two specific mRNA species are responsible

for the two forms. I have done this by carrying out preliminary *in vitro* translation studies on RNA from various stages of the *D. discoideum* developmental cycle.

3.3 Results

My initial attempts to translate *Dictyostelium* RNA *in vitro* were met with little success. I eventually purchased an *in vitro* translation kit from Bathesda Research Laboratories, and using the kit I was able to obtain efficient translation of both commercial RNA preparations and *Dictyostelium* total RNA. I decided to use poly-A RNA for all future translations in the hopes of increasing the amount of phosphorylase mRNA that could be translated in each reaction. Poly-A RNA was obtained from six developmental stages, as described under Methods and Materials, and these RNAs were also translated efficiently by the BRL lysate. Trichloroacetic acid (TCA) precipitated proteins from these translations routinely gave over 50-fold stimulation above endogenous lysate translation. Figure 10 shows the kinetics of ^{35}S incorporation into TCA-precipitable proteins translated from amoebae poly-A RNA; incorporation was linear up to 0.5 μg of exogenously added RNA. Similar results were obtained from other developmental stages.

I next turned my attention to immunoprecipitation of the translation products. Immunoprecipitations of the translation products of slug RNA with anti-'a', followed by SDS-PAGE and fluorography, revealed a protein doublet of 100-110 kd that was specifically precipitated by the antibody (Figure 11A). Anti-'b' immunoprecipitations, carried out on poly-A RNA from the amoeba stage, seemed to result in many labelled proteins being precipitated. However, only two of these proteins, with molecular weights of 60 kd and 92 kd, were specifically recognized by the antibody; the other proteins appeared to be non-specifically precipitated by the Protein-A-Sepharose used in the immunoprecipitation protocol (Figure 11B).

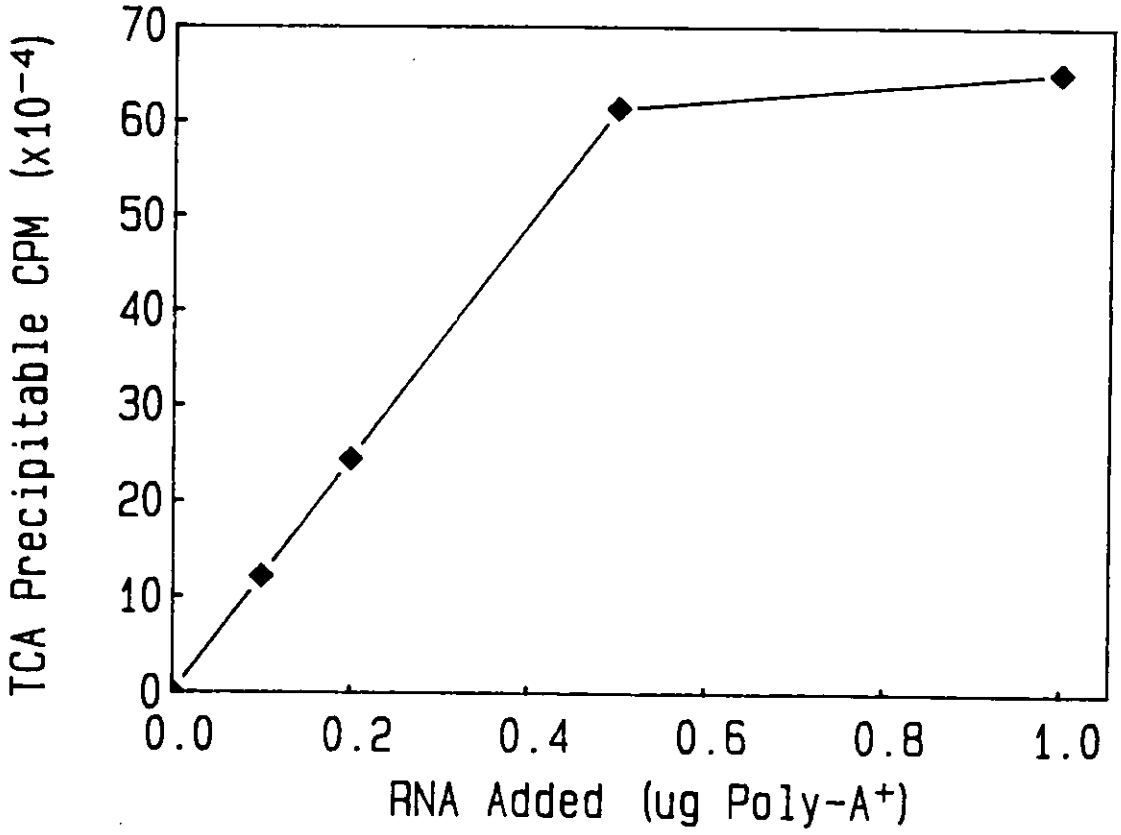


Figure 10. **Incorporation of ^{35}S-methionine into protein synthesized from in vitro translation of amoeba poly-A RNA:** Amoeba poly-A RNA was translated *in vitro* in a rabbit reticulocyte lysate with 20 uCi ^{35}S-methionine per reaction. A 2.0 ml sample of the translation products from each reaction was subjected to TCA precipitation onto fiberglass filters. The filters were dried and inserted into scintillation vials with 4.0 ml scintillation fluid; counts were obtained with a liquid scintillation counter.

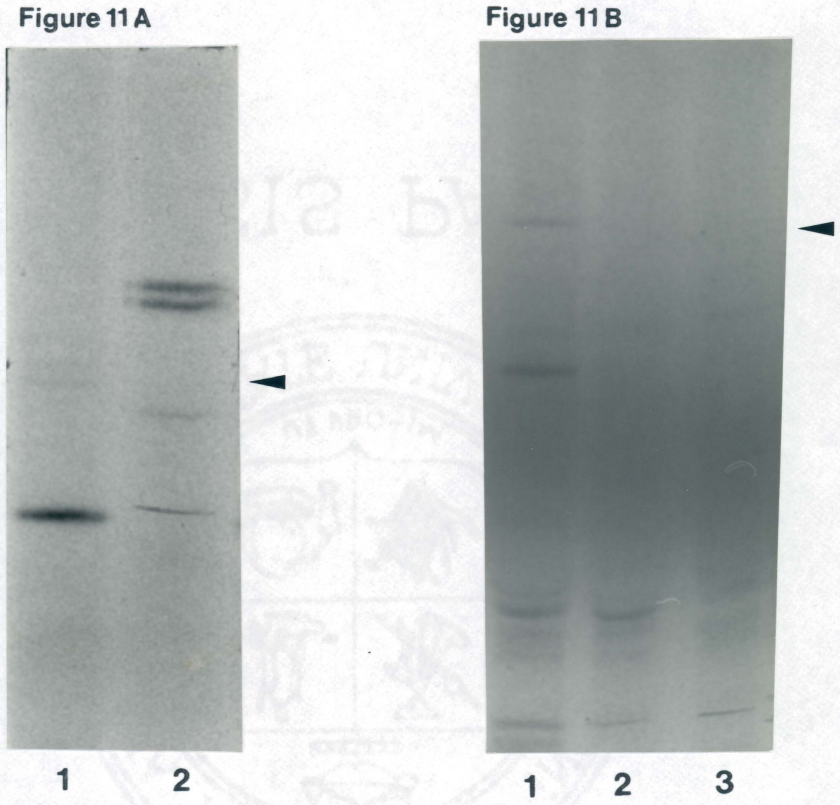


Figure 11. **Immunoprecipitation of in vitro translation products with anti-'a' and anti-'b' antisera:** Poly-A RNA from amoeba and slug stages was translated *in vitro* and translation products were immunoprecipitated. Figure 10A: Immunoprecipitation of translation products of slug RNA. **Lane 1**, preimmune serum; **Lane 2**, anti-'a'. Figure 10B: Immunoprecipitation of translation products of amoeba RNA. **Lane 1**, anti-b; **Lane 2**, preimmune serum; **Lane 3**, no antiserum (Protein-A-Sepharose only). Arrows indicate 92 kd.

The results of the immunoprecipitations shown in Figure 11 were consistent with the evidence, presented in Chapter 2, that suggested the two proteins are of different molecular weights; the anti-'b' precipitated a protein of 92 kd from an early developmental stage, and the anti-'a' precipitated a protein of higher molecular weight from a later developmental stage. These results also indicated that full length polypeptides for the two forms were being synthesized in the *in vitro* translation system. I then turned to examining the developmental expression of the mRNA(s) for the two forms of glycogen phosphorylase. Stage studies were carried out to examine the developmental expression of the mRNA(s); that is, poly-A RNA from the six developmental stages was translated *in vitro*, the translation products were immunoprecipitated with anti-'a' or anti-'b', and carried through SDS-PAGE and fluorography. Figure 12A shows the results of the stage study in which the translation products were immunoprecipitated with anti-'b'. Three proteins were immunoprecipitated by anti-'b'. In the early developmental stages, a 60 kd protein was much more intensely immunoprecipitated than was a 92 kd protein. In the later developmental stages the reciprocal was observed; the 92 kd protein was immunoprecipitated much more intensely from these stages. The third protein which was immunoprecipitated by the anti-'b' was the 100-110 kd protein. This protein was first immunoprecipitated from the translation products of the aggregation stage RNA and persisted through the remaining developmental time points. It is interesting to note that the banding pattern of these immunoprecipitated proteins almost exactly matched the banding pattern seen in the stage study shown in Chapter 2 (Figure 2A). In that stage study, anti-'b' was used to stain a western blot of relatively crude cell extracts from seven developmental stages. Figure 12B shows the results of the stage study in which translation products were immunoprecipitated with anti-'a'. The major protein immunoprecipitated by anti-'a' was the 100-110 kd protein; however, a doublet was seen in this case. Again, this protein (doublet) was first detected in the translation products of aggregation stage RNA and, again, persisted thereafter. The banding pattern of the 100-110 kd protein seen in these immunoprecipitations matched the banding pattern exhibited by the 104 kd protein seen in the stage study in Chapter 2 (Figure 2B), where anti-'a' was used to stain a western blot of relatively crude cell extracts from seven developmental stages. These re-

sults suggest that two distinct mRNA species are responsible for the two forms of glycogen phosphorylase in this organism.

3.4 Discussion

The stage study data presented above strongly suggest that two separate mRNA species exist for the two forms of glycogen phosphorylase in *Dictyostelium*. The anti-'b' stage study shows that the 92 kd protein is translated *in vitro* from RNA of every developmental stage. This suggests that the mRNA for the 'b' form is present throughout development. The identity of the 60 kd protein is not known; it may be a degradation product of the 92 kd protein (which the antibody obviously recognizes), or it may be a cross-reactive antigen. It is tempting to speculate that the 60 kd protein represents a degradation product of the 92 kd 'b' enzyme, particularly since these two proteins show reciprocal banding patterns in the stage study. However, I have no evidence to support this possibility and it is just as likely that the 60 kd protein represents a cross-reactive antigen. Still another possibility is that the 60 kd protein represents the translation product of a truncated phosphorylase 'b' mRNA. The anti-'b' stage study also shows that the 100-110 kd protein is translated only at later developmental stages; this suggests that a separate mRNA, which is not present at every developmental stage, exists for this protein. The anti-'a' stage study also suggests that the 100-110 kd protein is the product of a developmentally regulated mRNA; however, the anti-'a' immunoprecipitated a doublet of 100-110 kd, whereas only a single band was immunoprecipitated by the anti-'b'. The significance of the singlet versus the doublet is unclear. It is very likely that the 100-110 kd protein represents phosphorylase 'a', although there is a slight molecular weight discrepancy seen when comparing the immunoprecipitated translation product with the 'a' enzyme immunoprecipitated from DE52 column fractions. The 'a' enzyme immunoprecipitated from column fractions had a molecular weight of 104 kd (see Chapter 2, Figure

Figure 12A

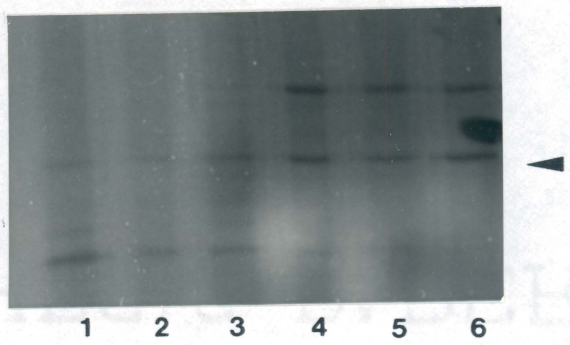


Figure 12B

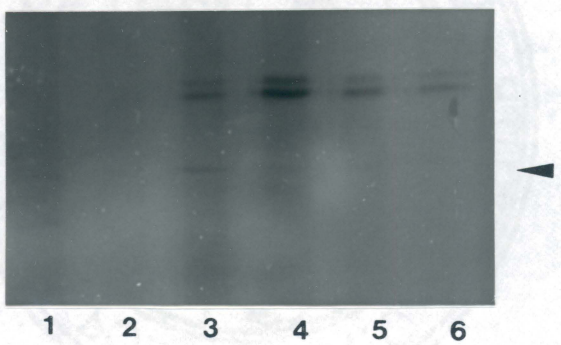


Figure 12. Stage study analysis: Immunoprecipitations of in vitro translation products from six developmental stages: Poly-A RNA from six developmental stages was translated *in vitro*; translation products were immunoprecipitated with anti-'b' (shown in Figure 12A) or anti-a (shown in Figure 12B). In both figures the RNA was from the following stages: **Lane 1,** amoebae; **Lane 2,** rippled amoebae; **Lane 3,** aggregates; **Lane 4,** early slugs; **Lane 5,** migrating slugs; **Lane 6,** culmination. Arrows indicate 92 kd.

5) whereas the immunoprecipitated translation product often showed a molecular weight of 110 kd. This discrepancy could result if the 'a' enzyme is initially synthesized as a larger, precursor molecule. Higgins and Dahmus (1982) have reported the *in vitro* translation of larger, precursor forms of glycogen phosphorylase 'a' from *Dictyostelium* RNA under certain conditions.

While the data described above do, as mentioned, strongly suggest that two mRNAs are responsible for the two forms of glycogen phosphorylase in this organism, conclusive evidence has not yet been obtained. A single message may still be responsible, with a single translation product that is proteolyzed to give the 92 kd and 100-110 kd proteins. Another possibility is that a single mRNA for phosphorylase is present and that the molecular weight difference between the two forms is due to phosphorylation; I have discussed this possibility earlier (Chapter 2). If phosphorylation is the reason for the observed molecular weight difference, an appropriate kinase or kinases must be present and functioning in the translation reactions, since the molecular weight difference between the two forms is observed in the translation experiments. I feel this is rather unlikely. Conclusive proof of two mRNA species awaits the results of hybrid-arrested and hybrid-selected translation experiments which are currently in progress. Also in progress are peptide mapping experiments which should allow me to determine if the immunoprecipitated translation products are in fact glycogen phosphorylase 'a' and 'b'.

I should point out that even if distinct mRNAs are conclusively shown to exist for the two forms of phosphorylase this would not immediately indicate that there are two genes for this enzyme in *Dictyostelium*, for it has been shown that in some other eukaryotic systems a single transript can be differentially spliced to yield distinct mRNAs (Early et al., 1980; Young et al., 1981). Indeed, some evidence suggests that this type of phenomenon occurs in *Dictyostelium*. In *Dictyostelium*, two developmentally regulated forms of UDP-glucose pyrophosphorylase have been identified (Fishel et al., 1982) and these two forms appear to have distinct mRNAs (Fishel et al., 1985). However, Dimond et al. (1976) were able to abolish expression of all UDP-glucose pyrophosphorylase activity with a single mutation; these data suggested that a single gene for UDP-glucose pyrophosphorylase exists, although Fishel et al. (1982) point out that the mutation may have altered a common regulatory gene or an essential subunit for both forms of the enzyme.

Thus, while it appears likely that separate mRNAs exist for the two forms of glycogen phosphorylase in *Dictyostelium*, the question of whether one or two genes exist for this enzyme is far from resolved.

3.5 Methods and Materials

Materials

Oligo-dT cellulose was purchased from Boehringer Mannheim, and ^{35}S-methionine was purchased from New England Nuclear. Unless otherwise indicated, all other materials were purchased from Sigma Chemicals.

RNA Extraction and Oligo-dT Cellulose Columns

RNA was extracted as described by Kimmel and Carlisle (1986). Oligo-dT cellulose columns were run as described in Maniatis et al. (1982).

In Vitro Translation

All of the components needed for the *in vitro* translations were provided in the kit purchased from BRL; translation reactions were run exactly as described in the BRL protocol. Final ionic conditions in the translation reactions were as follows: 1.2 mM $MgCl_2$, 17 μM EDTA, 40

mM KCl, 23.3 mM NaCl, 170 μM dithiothreitol, 8.3 μM hemin, 17 μg/ml creatine kinase, 0.33 mM $CaCl_2$, 0.67 mM EGTA, 33 mM creatine phosphate, 19 amino acids (minus methionine) at 170 μM each, 87 mM potassium acetate, and 25 mM HEPES at pH 7.2. When poly-A RNA was translated, 1.5 μg calf liver tRNA (Boehringer Mannheim) was added per reaction. All RNA was added to each translation reaction in volumes of sterile, distilled water. Each reaction contained 20 μCi ^{35}S-methionine. Each reaction was carried out in Eppendorf microcentrifuge tubes which had been autoclaved and treated with diethylpyrocarbonate (DEPC).

Quantitative Analysis of Translation Products By TCA Precipitation

Translation efficiency was analyzed by determining ^{35}S incorporation into TCA precipitated proteins. A 2.0 μl sample of each translation reaction was added to a tube containing 2.0 ml 0.5 M NaOH and 0.7% hydrogen peroxide and incubated at 37° C for 15 min. After this incubation, 1.0 ml ice cold 25% TCA was added and the tubes were incubated on ice for 10 min. The samples in these tubes were then placed into the wells of a vacuum filtration manifold (Millipore) containing a 24 mm fiberglass filter (Boehringer Mannheim); filters were always presoaked in ice cold 8% TCA. Precipitated proteins were trapped on the filters as the fluid was drawn into the manifold under vacuum. The filters were then washed twice with 5 ml volumes of ice cold 8% TCA, removed from the manifold well, and dried under a heat lamp. The dried filters were placed into scintillation vials, 4 ml scintillation fluid (Ecoscint, National Diagnostics) was added, and the radioactivity was determined using a Tm Analytic Model 6895 liquid scintillation counter.

Immunoprecipitation of Translation Products

The translation products were immunoprecipitated by placing the Eppendorf tubes on ice to stop the translations; translation reaction volumes were brought up to 100 μl with the addition of 70 μl RIPA buffer (10 mM Tris-HCl at pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1% NP40, 0.5% deoxycholate, and 0.1% SDS). Antiserum was added (10 μl per tube) and the tubes were incubated for 1 hr at 4° C; 20 μl Protein-A-Sepharose (at 80 mg/ml) was added and the tubes were incubated, with shaking, for an additional 30 min. The tubes were then centrifuged for 30 sec in an Eppendorf microcentrifuge. Supernatants were removed and the pellets were washed with 1.0 ml RIPA buffer containing 0.5 M NaCl. The pellets were then washed four more times, as follows: washes 1 and 2, 1.0 ml each with RIPA; washes 3 and 4, 1.0 ml each with TAB buffer (50 mM Tris-HCl at pH 7.5, 2 mM benzamidine, 0.02% sodium azide). After the final wash the pellets were resuspended in 50 μl TAB containing 5% DTT and 2% SDS, boiled for 3 min, and centrifuged once again; the resultant supernatants were used for SDS-PAGE.

Fluorography

SDS gels were stained for protein with silver to identify molecular weight markers. The silver stained gels were impregnated with Amplify fluorographic reagent (Amersham) by soaking them in the Amplify for 25 min. The gels were then dried under vacuum at 80° C and were exposed to Kodak X-OMAT RP film; film exposure was usually 24-72 hrs at -70° C. Film was developed using Kodak GBX developer and fixer.

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