Protein Phosphatases and Protein Kinases in Dictyostelium

by

Douglas K. Ferris

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APPROVED:

Charles L. Rutherford, Chairman

F. M. Anne McNabb

Allan A. Yousten

George W. Claus

Eugene M. Gregory

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(ABSTRACT)

In the present work, the chromatographic behavior and partial characterization of 5 protein phosphatases, 6 paranitrophenyl phosphate (pnpp) phosphatases and 2 protein kinases from *Dictyostelium* are described. Two of the protein phosphatases are shown to have subunit structures. All of the protein phosphatase activities described were able to dephosphorylate sites on proteins that had previously been phosphorylated by the cAMP-dependent protein kinase. Therefore it may be that these phosphatases function *in vivo* to antagonize the action of the cAMP-dependent protein kinase. Various pnpp phosphatase activities, varying in size, cation requirements and pH optima are described. Since many pnpp phosphatases also function with protein substrates it is possible that these activities represent protein phosphatases that function with phosphoproteins that have not been tested.

Evidence for the existence of protein kinase C in *Dictyostelium* is presented. A histone protein kinase was found in *Dictyostelium* extracts that eluted from DE52 cellulose at the same salt concentration that rabbit brain protein kinase C did. In addition in one experiment clear dependency on calcium and phospholipids is shown.

The purification to homogeneity, and characterization of a low molecular weight autophosphorylating protein kinase, pp20, is described. This protein kinase is a major phosphorylated protein and in addition represents at least 0.03% of the total cellular protein. The autophosphorylation reaction can be inhibited by EDTA but not by EGTA. Following inhibition by EDTA, autophosphorylation can be reactivated by the addition of Mn^{2+}, Mg^{2+}, or Ca^{2+}. Western blotting evidence is presented that shows cross reactivity of pp20 and an 85 KDa protein that may possess casein kinase activity.

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First I would like to thank Dr. Rutherford for giving me a position in his laboratory and particularly, for allowing me a large measure of independence in my research. He has, for the most part, let me use my own judgement in deciding what questions to pursue, and how to pursue them, and I only hope he is not too dissatisfied with the results. For my part, although I learned many different scientific techniques while here, I will remember Dr. Rutherford's personal example of patient steady work, towards clearly defined goals, as my most valuable training.

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Lastly I am deeply grateful to my family. I thank my wife , who has worked a numbing schedule as both a laboratory technician and mother, and , who do not understand, but continue to love me anyway.

Table of Contents

List of Illustrations

List of Tables

1. Introduction

Yeats wrote that "the fascination with what is difficult" had drawn him to his craft (Yeats, 1933). He might well have been writing about the study of development. At issue is the question of how a single cell can reproduce and grow into an adult organism composed of many different organs, tissues and cell types. What are the signals that turn appropriate genes on or off at precisely the right moment in precisely the right cells? How are morphogenic movements during pattern formation guided? What mechanisms operate to modulate cell growth so that only the necessary numbers of cells are produced? Finally, how is cell death programmed, so that certain groups of cells are sacrificed to further the developmental scheme?

Development in higher metazoans is obviously a tremendously complex operation during which many separate processes occur simultaneously. Cells grow, divide, move and differentiate all at the same time, and in a mammal there are hundreds of organs and cell types. Elucidating all of the underlying biochemical mechanisms regulating mammalian development is currently a complicated task. The question then, is how to most fruitfully study growth and development?

It has often proven the case that to learn something about a complicated system, the best approach is to study a simpler system in which a similar process occurs.

1.1 Dictyostelium discoideum, a model for development.

Since the discovery of *Dicyostelium discoideum* by Raper (Raper 1935) this organism has become a frequently used system for the study of development. Several factors contribute to its wide usage. *Dictyostelium* are easy to grow in the laboratory and synchronous development of the genetically identical cells can be achieved by removing the food source. Since the development of the organism does not begin until the onset of starvation, the growth and differentiation stages are separated in time and can be studied as discrete processes. Perhaps the major advantage of the system however, is that only two cell types are formed and they are separated spatially. This is in marked contrast to mammals, in which even a single organ contains many different cell types which are not easily separable.

1.1.1 The life cycle of Dictyostelium discoideum

In nature *Dictyostelium* amoebae inhabit forest floor leaf litter where they feed on bacteria. While conditions remain favorable the amoebae grow and reproduce by binary fission. However, if the food source becomes depleted, the amoeba begin to aggregate into groups containing anywhere from 10,000 to 100,000 cells (Loomis, 1975). The process of aggregation starts when individual cells autonomously begin to excrete an acrasin or chemoattractant. In 1968 Konijn and his co-workers discovered that the heat stable acrasin was cyclic adenosine monophosphate (cAMP). The amoeba move towards the cell and themselves begin emitting periodic pulses of cAMP which diffuse outward to attract a*Dicyostelium discoideum*itional cells (Shaffer, 1975). After aggregation, the cells go through a series of morphogenic movements and eventually form a small fruiting body composed of a slender stalk supporting a small spore head Figure 1 on page 4. The cells which comprise the stalk age and eventually die but the spores remain viable and are released

to reinitiate the cycle. In this simple system all of the major elements of mammalian development are present; growth, differentiation, morphogenesis, pattern formation, senescence and death. While the development of *Dictyostelium* is intrinsically interesting, probably most of those who work with this organism do so in the belief that many of the fundamental mechanisms regulating its growth and development are similar to those in mammals.

1.2 Cyclic phosphorylation, a fundamental regulatory mechanism

During the past ten years it has become clear that cyclic phosphorylation and dephosphorylation of proteins is a fundamental mechanism for regulating many cellular activities. The cellular functions in which cyclic phosphorylation is now known to be involved include glycogen metabolism, glycolysis, gluconeogenesis, lipid metabolism, steroid synthesis, protein synthesis, mitotic chromosome condensation, and control of growth and differentiation.

In the late fifties Sutherland and his colleagues (Rall et al. 1957; Rall & Sutherland 1958) demonstrated that epinephrine stimulated gluconeogenesis by increasing the synthesis of cyclic adenosine monophosphate (cAMP). At that time there was no understanding of how a hormone or neurotransmitter might cause changes in specific target cells. While cAMP quickly became implicated in a wide variety of biological processes, the mechanism by which cAMP acts remained unknown for a decade.

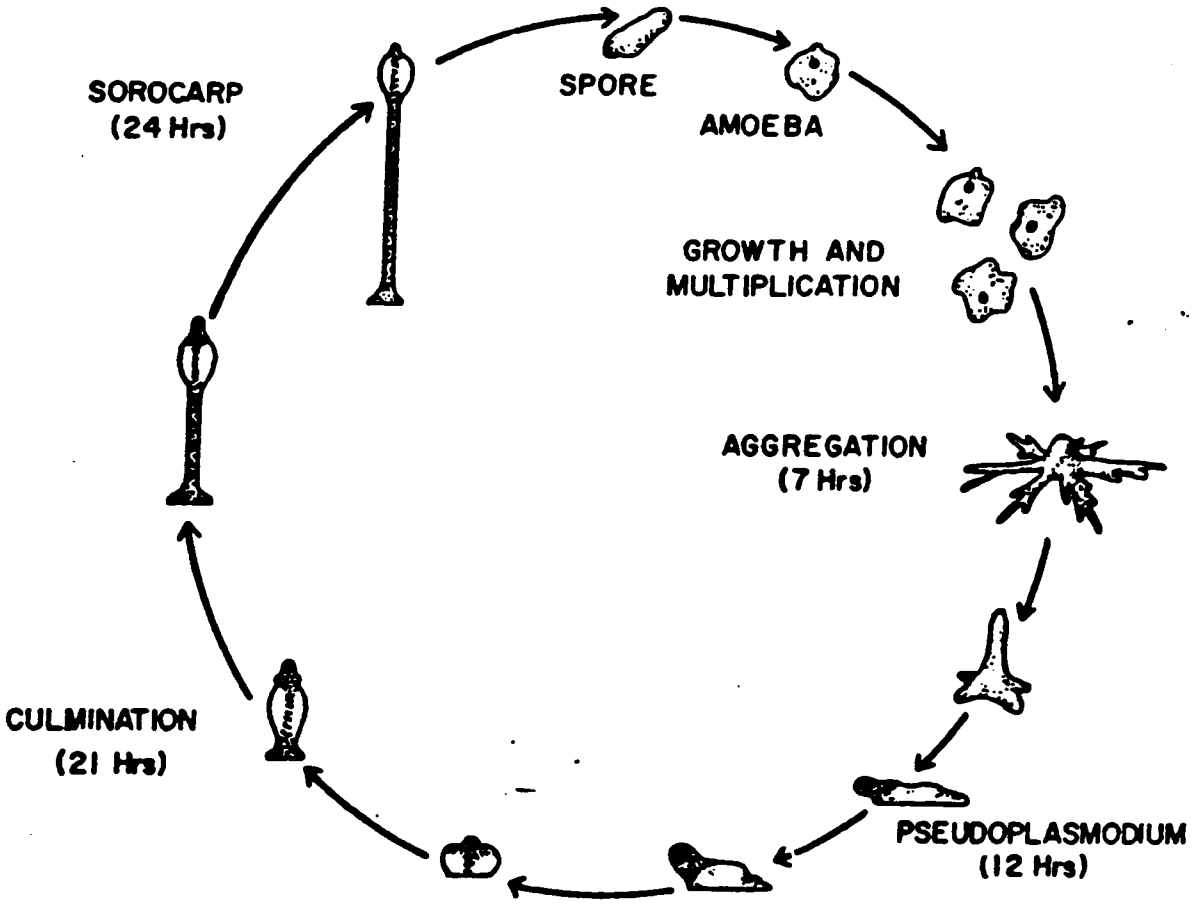

Fig.1. THE DEVELOPMENTAL CYCLE OF
Dictyostelium discoideum

Figure 1. Dictyostelium life cycle

1.3 Protein kinases

1.3.1 The cAMP-dependent protein kinase

In 1968 Krebs and co-workers (Walsh et al. 1968) discovered a cAMP dependent protein kinase in skeletal muscle and provided evidence that its activation by cAMP was sufficient to cause synthesis of glycogen. The following year it was found that cAMP dependent protein kinase is present in many tissue types and is virtually ubiquitous throughout the phyla of the animal kingdom (Kuo and Greengard, 1969). These findings led to the hypothesis that all of the many effects of cAMP in eukaryotes are mediated solely through the action of the cAMP dependent protein kinase.

The protein kinase hypothesis states that a hormone which acts through cAMP, binds to its receptor, activating adenylate cyclase, the cAMP synthetic enzyme. The newly synthesized cAMP binds to, and activates, the cAMP dependent protein kinase which begins to phosphorylate its substrate proteins. These substrate proteins then either directly or indirectly, through subsequent enzymatic steps, bring about the characteristic response of the particular hormone. This central paradigm has been amply supported by research conducted during the last ten years. In mammals and other higher metazoans the regulatory subunit of the cAMP dependent protein kinase is the only known cAMP binding protein (Walter & Greengard, 1982). It has been shown that mutants of some mammalian cell lines which are deficient in cAMP dependant protein kinase are unable to respond normally to hormones which act through cAMP (Coffino and Gray 1978; Gutmann et al. 1978; Costa et al. 1982). In other elegant experiments the catalytic or regulatory subunit of the kinase has been microinjected into various types of cells. In the amphibian oocyte, progesterone stimulates meoisis, apparently by decreasing intracellular concentrations of cAMP. The stimulation of meoisis by progesterone is blocked by injection of the catalytic subunit, while injection of the

regulatory subunit of the cAMP-dependent protein kinase (thereby inhibiting the catalytic subunit) mimics the action of progesterone (Maller & Krebs 1977). In mammalian heart muscle cells, cAMP regulates the conductance of voltage dependent calcium channels. Injection of the catalytic subunit mimics the regulation by cAMP and again, injection of the regulatory subunit blocks the effects of cAMP (Osterrieder et al. 1982). In mammalian smooth muscle, cAMP acts as a relaxant, inhibiting calcium induced tension. Injection of the catalytic subunit also inhibits calcium induced tension (Kerrick & Hoar 1981). Recently, Schimmer and co-workers (1986) recovered normal hormonal regulation in adrenal cells that contained a defective cAMP-dependent protein kinase, by transfer of a kinase gene. The accumulation of this type of evidence strongly indicates that the action of the cAMP-dependent protein kinase is both necessary and sufficient to explain the effects of cAMP in higher metazoans.

While the discovery of the cAMP dependent protein kinase allowed the development of a conceptual framework explaining how some hormones and neurotransmitters elicit intracellular responses, it is only one of an entire class of phosphorylating enzymes.

1.3.2 The cGMP-dependent protein kinase

Shortly after the cAMP dependent enzyme was found, a cyclic guanosine monophosphate (cGMP) dependent protein kinase was discovered and shown to be present in many tissues of diverse animal phyla (Greengard & Kuo 1970; Kuo et al. 1971; Walter 1981). It is not unreasonable, by analogy with the cAMP system, to expect that the cGMP-dependent protein kinase mediates most of the varied effects of cGMP on cellular functions.

1.3.3 Calcium/calmodulin-dependent kinases

The discovery of the calcium/calmodulin-dependent protein kinases followed in 1978 (Dabrowska et al. 1978; Schulman & Greengard 1978 b; Yagi et al. 1978). The members of this subgroup require both calcium and the highly conserved calcium binding protein calmodulin for optimal activity. At present, four distinct calcium/calmodulin-dependent protein kinases have been identified, myosin light chain kinase (Dabrowska et al. 1978; Yagi et al. 1978), phosphorylase kinase (Cohen et al. 1978) and two different enzymes isolated from brain tissue (Kennedy & Greengard, 1981).

1.3.4 Calcium/phospholipid-dependent kinase

In 1979 Takai and co-workers discovered an additional calcium activated protein kinase, however this enzyme requires phospholipids, specifically phosphatidylserine, rather than calmodulin (Takai et al.1979a; 1979b). In the few years since its discovery, the calcium/calmodulin-dependent kinase has become recognized as an important intracellular mediator of a wide variety of extracellular signals that cause breakdown of phosphatidylinositol and production of diacylglycerol (Nishizuka 1984).

1.3.5 Tyrosine kinases

A fourth major subgroup of protein kinases are transmembrane proteins which are regulated by such molecules as insulin (Kasuga et al. 1982), epidermal growth factor (Rubin et al. 1982), transforming growth factor (Reynolds et al. 1981) and other growth factors (Jacobs et al. 1983). The members of this category phosphorylate tyrosine residues on specific substrate proteins, in

contrast to other protein kinases which phosphorylate serine and threonine residues (Collett et al. 1980). Some of these tyrosine kinase activities are involved in growth regulation (Rubin et al. 1982), and aberrant expression or activity may lead to cell transformation (Nakamura et al. 1983). Several members of this class of kinase activities are proto-oncogenes from which viral oncogenes, such as the src gene of the Rous sarcoma virus, were derived (Collett et al. 1980;see Kolata 1983; also Hunter 1984).

1.3.6 e1f-2 kinases

Two other important kinase activities have been identified which do not fit into any of the groups previously discussed. While one is a double stranded RNA dependent protein kinase and the other is inhibited by heme, both can inhibit the initiation of protein synthesis by phosphorylating protein synthesis initiation factor e1f-2 (Ranu 1982 a & b; Traschel et al. 1978).

1.3.7 Kinases of unknown regulation and function.

In addition to those kinases mentioned above, where something is known of their functions or regulatory mechanisms, others have been described, which are regulated by unknown factors, and whose functions remain a mystery.

1.4 Protein Phosphatases

The discussion above has been restricted to protein kinases, but they constitute only one half of the reversible phosphorylation system. The protein phosphatases have been less well studied than the protein kinases, for a variety of reasons. There has been a tendency to regard phosphorylation as being more important and better regulated than dephosphorylation. However, the phosphorylation state of a protein which is regulated by phosphorylation, is controlled by a dynamic equilibrium between the action of a kinase and the action of the opposing phosphatase. Clearly both types of enzymes are required and they are equally important. It was also thought for some time that protein phosphatases were less specific than protein kinases. While it is true that some of the phosphatases exhibit relatively broad substrate specificity *in vitro*, others are remarkably specific. It may also be that purified preparations of those enzymes which dephosphorylate many different substrates in vitro, have lost regulatory components which confer specificity. This idea is supported by the fact that in crude preparations many phosphatases exhibit molecular weights in excess of 200 kilodaltons while most of the purified enzymes have molecular weights in the 30-40 kilodalton range (see Ingebritsen & Cohen 1983 for a review). The size variability of these activities during purification has been a major difficulty both in terms of purification and in terms of understanding their regulation (Kato & Sato 1974; Kobayshi et al. 1975; Kobayshi & Kato 1977). It has also made comparison of the various enzyme preparations from different laboratories problematic. These difficulties notwithstanding great strides have been made towards determining the number and specificity of these enzymes.

1.5 Four types of protein phosphatases

The protein phosphatases, are characterized by a variety of criteria including size, substrate specificity, cation requirements and differential sensitivity to low molecular weight inhibitor proteins. It is now known that four enzymes, called protein phosphatases 1, 2A 2B, and 2C are responsible for regulating most of the phosphoproteins involved in glycogen metabolism, glycolysis, gluconeogenesis, fatty acid synthesis, cholesterol synthesis, and protein synthesis (for review see Ingebritsen & Cohen 1983 a). Protein phosphatase 1 selectively dephosphorylates the beta subunit of phosphorylase kinase and can be inhibited by extremely low concentrations of inhibitor 1 and inhibitor 2. The type 2 enzymes, on the other hand, preferentially dephosphorylate the alpha subunit of phosphorylase kinase and are not inhibited by either inhibitor 1 or inhibitor 2 (Ingebritsen & Cohen 1983 a).

1.5.1 Protein phosphatase 1

The regulation of protein phosphatase 1 is interesting and complex. It can be isolated in an inactive form which requires preincubation with MgATP to become activated. This inactive form consists of a catalytic subunit complexed with inhibitor 2. The activation event involves phosphorylation of inhibitor 2 by glycogen synthase kinase (Hemmings et al. 1981). Protein phosphatase 1 is also regulated by inhibitor 1 in a cyclic AMP-dependent manner. Inhibitor 1 is only functional as an inhibitor following phosphorylation by the cAMP-dependent protein kinase (Nimmo & Cohen 1978). Recently it has been demonstrated that in vitro phosphorylation of protein phosphatase 1 by pp60 V-src caused a 39% reduction in its phosphatase activity (Johansen & Ingebritsen 1986). Thus the activity of protein phosphatase 1 is coordinately regulated by two inhibitor proteins, and at least two, and possibly three, different protein kinases. Such a complex regulatory mechanism suggests the importance of this enzyme in controlling cellular metabolism.

It is also interesting to speculate on the involvement of protein phosphatase 1 in cell transformation given its phosphorylation by pp60 V-src.

1.5.2 Type 2 protein phosphatases

Three type 2 enzymes, called 2A, 2B, and 2C have been identified as separate enzymes (Ingebritsen and Cohen, 1983; Ingebritsen et al.,1983; Pato et al. 1983; Stewart et al.1983). At least three forms of protein phosphatase 2A have been resolved but all of them apparently contain identical catalytic subunits (Ingebritsen et al. 1983). The type 2B enzyme was originally identified as a Mn^{2+} requiring, alpha phosphorylase phosphatase (Cohen 1978), but it is now recognized as a calcium/calmodulin dependent protein phosphatase (Stewart et al. 1982; Yang et al. 1982; Stewart et al. 1983). It is similar and perhaps identical to the neural calmodulin binding protein called calcineurin which has been demonstrated to have protein phosphatase activity (Stewart et al. 1982; Yang et al. 1982). Protein phosphatase 2B seems to have a relatively restricted range of substrates having demonstrated activity only on inhibitor 1, the alpha subunit of phosphorylase kinase and the P light chain of myosin (Ingebritsen & Cohen 1983).

Protein phosphatase 2C purified from rat liver has been shown to be a magnesium requiring enzyme with a broad substrate specificity (Ingebritsen & Cohen 1983; Ingebritsen et al. 1983; Pato et al. 1983). When purified to homogeneity it consisted of a single polypeptide of 43 kilodaltons.

The protein phosphatases discussed above are thought to account for most of the phosphatase activity in mammalian tissues. These enzymes, in conjunction with the protein kinases, form the regulatory basis for a wide range of cellular functions.

1.6 Interactions of phosphorylation/dephosphorylation systems

Since cyclic phosphorylation is an important means of regulating virtually every major cellular function, it is perhaps not surprising that the protein kinases and phosphatases should themselves be the objects of such regulatory mechanisms. It turns out that many protein kinases are phosphorylated either by other kinases or by autocatalysis, and both inhibitor 1 and inhibitor 2, which can be considered as subunits of protein phosphatase 1, can also be phosphorylated. It is by such mechanisms that many extracellular signals are amplified and coordinately expressed within the cell. The interactions of the enzymes involved in glycogen metabolism are the best understood so it is instructive to examine this system in some detail. In muscle, glycogen metabolism is regulated by adrenaline and insulin as well as by neural stimulation. Both muscle contraction and adrenaline elicit glycogen breakdown while insulin stimulates glycogen synthesis. They cause these changes by altering the activities of glycogen synthase and glycogen phosphorylase the glycogen synthetic and degradative enzymes respectively. When adrenaline is bound by receptors on the plasma membrane, the activity of adenylate cyclase is transiently increased causing a rise in intracellular cAMP levels. The cAMP binds to, and activates, the cAMP-dependent protein kinase according to the following formula, where R is the regulatory subunit and C is the catalytic subunit (Greengard & Kuo 1970). R_2C_2(inactive) + cAMP -----> R_2-cAMP + 2C(active). The free catalytic subunit phosphorylates inhibitor 1, phosphorylase kinase and glycogen synthase (Cohen 1973;Cohen 1978;Soderling et al. 1977). Phosphorylation of inhibitor 1 causes it to become an active inhibitor of protein phosphatase 1, the enzyme responsible for dephosphorylation of glycogen synthase, phosphorylase kinase Beta subunit and phosphorylase "a" (Antoniw & Cohen 1976; Cohen et al. 1977;Ingebritsen et al. 1983). Phosphorylation by the cAMP-dependent kinase inhibits the activity of glycogen synthase but stimulates that of phosphorylase kinase. The activated phosphorylase kinase then phosphorylates phosphorylase "b", converting it to the active "a" form.

Thus binding of adrenaline by the cell surface receptors of muscle cells coordinately shuts down glycogen synthesis and stimulates glycogen breakdown to provide glucose for energy production.

Electrical stimulation of muscle contraction also causes glycogen degradation, but the pathway is different. In this case phosphorylase kinase is the primary mediating enzyme. The structure of phosphorylase kinase is (alpha,beta,gamma,delta)$_4$ where gamma is the catalytic subunit and delta is the calcium binding protein known as calmodulin (Soderling et al. 1979; Cohen et al. 1978; Chan & Graves 1982). When muscle cells are electrically stimulated Ca^{2+} is released from the sarcoplasmic reticulum and bound by the delta subunit causing stimulation of the catalytic subunit. Activated phosphorylase kinase has been shown to phosphorylate and inhibit the activity of glycogen synthase although it does not modify the same serine residues as the cAMP-dependent enzyme.

One additional kinase called glycogen synthase kinase phosphorylates glycogen synthase and it phosphorylates serine residues distinct from those modified by either the cAMP-dependent enzyme or phosphorylase kinase but it is unknown how this enzyme is regulated.

Protein phosphatase 2B, the calcium/calmodulin-dependent enzyme is also activated by the release of calcium during muscle contraction. This enzyme is capable of dephosphorylating the alpha subunit of phosphorylase kinase (both the alpha and beta subunits are phosphorylated by the cAMP-dependent kinase) as well as inhibitor 1 (Stewart et al. 1982; Stewart et al. 1983).

We can now begin to see how these various enzymes work together in a coordinated manner in muscle tissue to regulate glycogen metabolism. In addition a relationship between the cAMP-dependent and the calcium/calmodulin-dependent systems is suggested.

1.7 Autophosphorylation by protein kinases

As was mentioned in the previous section some, and perhaps most, protein kinases undergo autophosphorylation. In some cases such autophosphorylation is clearly a regulatory mechanism while in others the significance is not at all clear. The cAMP-dependent enzyme is known to autophosphorylate, and in this case the catalytic subunit phosphorylates the regulatory subunit (Erlichman et al. 1974). Phosphorylation of the regulatory subunit slows the rate at which the dissociated regulatory and catalytic subunits will reassociate when cAMP levels are reduced (Rangel-Aldao, & Rosen 1976). The functional effect is therefore to lengthen the duration of cAMP influence in the cell.

The cGMP-dependent protein kinase also autophosphorylates (De Jonge et al. 1977) by what is thought to be an intramolecular reaction (Lincoln et al. 1978). The evidence that the phosphorylation is intramolecular is that dilution over a wide range did not alter the rate or extent of phosphorylation. No effects of autophosphorylation by the cGMP-dependent kinase have yet been described.

The double-stranded RNA-dependent and the heme-regulated protein kinase inhibit protein synthesis by phosphorylating the same sites on elf-2. Both of these enzymes undergo autophosphorylation that seems to be a a requirement for enzyme activation (Ranu 1982 a & b; Trachsel et al. 1978).

1.8 Involvement of cyclic phosphorylation

in control of growth and differentiation

It has been mentioned several times that cyclic phosphorylation may be involved in the regulation of growth and development. Now it is appropriate to examine some of the evidence for that assertion. Because growth and differentiation must ultimately be regulated by the genome, phosphorylation of nuclear proteins is potentially very important.

1.8.1 Phosphorylation of histones

Histones are low molecular weight basic proteins that are associated with DNA in the nucleus. Histones are thought to function in maintaining the structure of chromatin and it has also been hypothesized that they are involved in gene regulation. Various workers have shown that stripped DNA supports more transcriptional activity than equivalent DNA complexed with histone and that the repression is proportional to the amount of histone bound (Huang and Bonner 1962; Spelsberg and Hnilica 1971). Phosphorylation of histone H1 has been reported in every tissue in which it has been examined (Hayashi & Iwai 1970; Sherod et al. 1970; Chae et al. 1972; Letmansky 1973). Both phosphoserine (Kleinsmith et al. 1966) and phosphothreonine are present with the rates of phosphorylation varying during the cell cycle (Hohmann et al. 1975). Phosphorylation of threonine seems to occur only at mitosis (Hohmann et al. 1976). Tryptic digests of histone H1 have shown that serine residue 37 on H1 is phosphorylated in a cAMP-dependent manner while serine 108 is phosphorylated by a cAMP-independent protein kinase (Adler et al. 1972). Histone phosphorylation is increased during periods of increased DNA and RNA synthesis (Gutierrez &

Hnilica 1967; Gutierrez-Cernosek & Hnilica 1971). Histone phosphorylation is also greater in rapidly dividing cells than in resting cells (Pearson & W. Kipaik 1972; Balhorn et al. 1972). It has also been shown that tumor growth rates correlate with the degree of histone phosphorylation (Balhorn et al. 1972) and that tumor promoters such as phorbol esters cause an increase in histone phosphorylation. One possibility which has been advanced is that phosphorylation of H1 causes chromosome condensation and initiates mitosis (Bradbury et al. 1973, 1974). Experiments showing that addition of a nuclear cAMP-independent protein kinase to *Physarum polycephalum* advances mitosis tend to support this idea (Inglis et al. 1976).

1.8.2 Phosphorylation of non-histone chromosomal proteins

While there are only a few types of histones and these do not show much tissue or cell type specificity, there are a great variety of non-histone chromosomal (NHC) proteins which do show tissue specificity (Smith & Chae 1973; Fujitani & Holoubek 1974). This heterogeneity may reflect a function in gene regulation in a tissue specific manner. It has been shown that addition of preparations of NHC proteins to intact chromatin causes an increase in RNA synthesis (Kamiyama & Wang 1971) and that new kinds of transcripts are produced. In an early experiment Gilmour and Paul (1970) prepared histones and NHC proteins from two different cell types and then added them to DNA prepared from a third tissue. They found that the the transcripts produced using this mixture as a template were the same as those produced in the tissue from which the NHC protein was derived.

It has been known since 1966 (Kleinsmith et al. 1966) that NHC proteins are phosphorylated, and it has been shown that both phosphorylation and dephosphorylation of NHC proteins occurs rapidly in the cell. Kamiyama et al. (1972) found that adding purified NHC which was phosphorylated, to DNA histone complexes, increased the ability of the complex to act as a

template for transcription. In the same study it was determined that the increase was proportional to the amount of phosphate incorporated into the NHC proteins.

At least 12 different protein kinase activities which phosphorylate NHC proteins have been resolved from nuclear extracts (Kish & Kleinsmith 1974) and none of these were active with histone as a substrate. While 5 of these kinases appeared to be stimulated by cAMP it was found that highly purified cAMP-dependent protein kinase was inactive towards NHC proteins. Others have shown cGMP stimulation of NHC phosphorylation in lymphocytes (Johnson & Hadden 1975).

1.8.3 Phosphorylation of RNA polymerase

All three nuclear RNA polymerases have been reported to be phosphorylated (Bell et al. 1976; Buhler et al. 1976; Bell et al. 1977) and phosphorylation has been reported to increase polymerase activity (Beebee 1973; Hirsch & Martello 1976).

1.9 Cyclic phosphorylation in Dictyostelium

1.9.1 The importance of cAMP

The effects of cAMP in *Dictyostelium* extend beyond its function as a chemotactic agent during aggregation. Intracellular levels of cAMP increase during aggregation and remain elevated during further multicellular development (Malkinson & Ashworth 1973; Pahlic & Rutherford 1979; Merkle et al. 1984). There is also evidence that cAMP is differentially distributed, with higher concentrations in the anterior prestalk cells (Bonner 1949; Brenner 1978; Rubin & Robertson

1975). Application of exogenous cAMP to *Dictyostelium* induces differentiation into stalk cells (Feit et al. 1978; Gross et al. 1981; Chia 1975). However an additional low molecular weight heat stable molecule called differentiation-inducing factor (DIF) is also required (Town et al. 1976; Kay & Jermyn 1983). Cyclic AMP in the absence of DIF induces spore cell differentiation but there may also be a requirement for cell contact (Kay et al. 1979; Landfear et al. 1981). The continued synthesis of late stage enzymes in disaggregated slugs depends on addition of cAMP (Gross et al. 1981; Kay et al. 1979; Landfear & Lodish 1980; Town & Gross 1978). It has also been shown that specific late stage mRNAs are rapidly degraded in disaggregated slugs, but are stabilized by addition of cAMP. In addition these mRNAs can be reinduced by cAMP after degradation has occurred in the absence of cAMP (Blumberg et al. 1982).

1.9.2 The cAMP-dependent protein kinase in Dictyostelium

After the discovery of the cAMP-dependent protein kinase in mammalian muscle tissue and the demonstration that cAMP was the chemoattractant in *Dictyostelium* considerable effort was devoted to identifying a similar enzyme in *Dictyostelium* (Weinstein & Koritz 1973). However, it was not until 1977 that a cAMP-dependent protein kinase from *Dictyostelium* was first reported (Sampson 1977), and confirmation of its presence by other groups took five more years (Rutherford et al. 1982; Leichtling et al. 1982; de Gunzburg & Veron 1982). The *Dictyostelium* enzyme has now been fairly well characterized (Cooper et al. 1983; Rutherford et al. 1984; Majerfeld et al. 1984; DeGunzburg et al. 1984) and its developmental regulation examined (Rutherford et al. 1982; Leichtling et al. 1984). The enzyme from *Dictyostelium* has physiochemical properties similar to the mammalian enzyme, however, only one isozyme of the regulatory subunit is present while two isozymes are found in mammals. The amount of the cAMP-dependent enzyme increases during development (Rutherford et al.1982; Leichtling et al. 1984) at the same time that cAMP levels also rise (Merkle et al. 1984). Using density gradient separated slug stage cells, Schaller and co-workers (1984) reported that the prespore cells contained 4-5 times the amounts of catalytic and regulatory

subunits than were found in the prestalk cells. In a later study using micro-dissected individuals (Vaughan & Rutherford 1986) found no differential distribution of the enzyme until very late stages when levels in stalk cells were one fourth of the levels found in the spore cells. Finally a very recent report describes the translocation into the nucleus of both the catalytic and regulatory subunits of the enzyme during development (Woffendin et al. 1986). In the same paper it was reported that there was a reverse translocation of the subunits back into the cytosolic fraction following dissagregation, and that subsequent addition of cAMP to the separated cells caused the re-entry of the subunits into the nucleus. These results suggest that the effects of cAMP on post aggregative development are mediated primarily through the action of the cAMP-dependent protein kinase and that by phosphorylating nuclear proteins it may directly alter transcriptional activities. This idea is supported by a study showing increases in, and alterations of phosphorylation patterns of *Dictyostelium* NHC proteins during development (Sinclair & Rickwood 1985).

1.9.3 Other kinases in Dictyostelium

While the cAMP-dependent kinase from *Dictyostelium* has been given a large amount of attention in recent years, little is known about other protein kinase activities in this organism. Weinstein & Koritz (1973) described a cAMP-independent protein kinase activity which they assayed either in intact cells or in crude broken cell preparations. The enzyme phosphorylated histone type 2, was not developmentally regulated and was optimally active with 3 mM magnesium as cofactor. The authors suggested that the enzyme might be associated with membranes since freeze thawing or sonication resulted in reduced activity. Juliani and Klein (1981) also described a membrane bound kinase activity which phosphorylated endogenous membrane proteins and which was not affected by cyclic nucleotides. Recently a nuclear cAMP-independent protein kinase was partially purified from amoebae and shown to phosphorylate threonine and serine residues of acidic proteins such as casein and phosvitin (Renart et al. 1984). The purified enzyme autophosphorylated using either ATP or GTP as phosphate donor and was strongly inhibited by

heparin suggesting that it can be classified as a type 2 casein kinase. For autophosphorylation to occur spermine had to be included in the reaction mixture, and its presence also stimulated the phosphorylation of casein. Spermine, however, inhibited the incorporation of phosphate into phosvitin. The purified enzyme produced a 38 KDa band after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), but the size of the native enzyme was not determined due to difficulties with aggregation during density gradient centrifugation.

While, with the exception of the cAMP-dependent protein kinase, little is known about protein kinase activities in *Dictyostelium*, even less is known about protein phosphatases. Until very recently the only mention of protein phosphatase activities from *Dictyostelium* occurred in protein kinase papers where the presence of phosphatase activities was regarded as a hindrance.

1.10 Rationale and objectives

It now seems likely that an explanation of development will involve learning about multiple phosphorylation/dephosphorylation systems. We have seen that none of these systems exists in complete isolation from the others within the living cell. Thus the effects of the cAMP-dependent system may be antagonized or enhanced by the actions of the cGMP-dependent, or other systems. Even in so simple an organism as *Dictyostelium* the actions and relationships of a variety of kinases and phosphatases must be understood. The present study was undertaken with the objective of learning about previously undescribed components of cyclic phosphorylation systems in *Dictyostelium* which may be involved in regulating development.

2. Protein Phosphatases in Dictyostelium discoideum

2.1 Abstract

We have examined protein phosphatase activities that are present during the cellular differentiation of *Dictyostelium*. Utilizing differential centrifugation, ion exchange, gel filtration and Concanavalin A affinity chromatography we found a number of distinct protein, acidic, neutral, and alkaline paranitrophenyl phosphate (pnpp) phosphatase activities. Three peaks of soluble Kemptide phosphatase activity and a very broad and heterogeneous soluble histone phosphatase activity were resolved by anion exchange chromatography. Histone, neutral and alkaline pnpp phosphatase activities were associated with the particulate fraction, while Kemptide phosphatase was not. The protein phosphatase activities were able to dephosphorylate sites that had been phosphorylated by the cyclic AMP dependent protein kinase. Therefore it is possible that their function *in vivo* may be to oppose the action of the cAMP dependent protein kinase. An apparent heat stable inhibitor of histone phosphatase is shown to be artifactual in that instead of interacting with the enzyme it acts by complexing with histone.

2.2 *Introduction*

During the last twenty years it has become clear that cyclic phosphorylation and dephosphorylation of proteins is a fundamental mechanism for regulating cellular metabolism (Walter and Greengard 1981). More recently, the importance of phosphorylation/dephosphorylation events to growth, differentiation and malignancy is becoming evident. Several growth factor receptors, cellular and viral oncogene products demonstrate protein kinase activity (Kolata 1983: Hunter 1984).

Cyclic adenosine monophosphate is known to be a regulator of development in *Dictyostelium.* Because the effects of cAMP in mammals are thought to be mediated entirely by cAMP dependent protein kinases (Corbin et *al.* 1975; Robinson et *al.* 1971) considerable effort was devoted to identifying such an activity in *Dictyostelium.* A cAMP dependent protein kinase in *Dictyostelium* has recently been reported by several groups (Rutherford et al. 1982: De Gunzberg et al. 1982; Cooper et al. 1982; Leichtling et al. 1982) which is similar to those described in mammals.

The protein phosphatases that are involved in the cyclic phosphorylation dephosphorylation reactions in mammals are much less well characterized than the kinases. However a number of protein phosphatases have been identified in mammalian muscle (Kato and Sato 1974; Kobayashi et al. 1975; Brandt et al. 1975; Antoniw and Cohen 1976; Cohen et al. 1977; Kobayashi and Kato 1977; Cohen 1978; Krebs and Beavo 1979; Lee et al. 1980; Li 1982; Ingebritsen and Cohen 1983a,b; Ingebritsen et al. 1983a,b,c; Pato et al. 1983; Stewart et al. 1983). Four types of phosphatases are thought to account for most of the protein phosphatase activity present in muscle tissue. These proteins range in size and complexity from large multi-subunit proteins of 260,000 daltons to isolated catalytic subunits of 35,000 to 38,000 daltons. These activities display variable substrate and cation specificities (Krebs and Beavo 1979; Lee et al. 1980; Li 1982; Ingebritsen and Cohen 1983a,b). While two reports concerning the cAMP dependent protein kinase in *Dictyostelium* briefly mentioned the presence of Kemptide phosphatase activity, (Cooper et al. 1983;Schoen et al.

1984) nothing additional has yet been published regarding the number or types of protein phosphatases that are present in this organism.

We report here on the resolution of one particulate and four soluble activities that are capable of dephosphorylating cAMP dependently phosphorylated substrates. It is therefore, possible that these protein phosphatase activities antagonize the effects of the cAMP dependent protein kinase *in vivo*.

2.3 Materials and Methods

2.3.1 Materials

[γ-^{32}P]ATP (25 Ci/mmol) was purchased from ICN. Kemptide was from Cal Biochem Behring. P 81 filter paper and DE52 cellulose were from Whatman, Sephacryl S300 from Pharmacia, Sephadex G100 and all other reagents were from Sigma.

2.3.2 Harvesting of Cells and Preparation of Extracts

Growth and differentiation of *D. discoideum* (NC 4) was carried out as previously described (Rutherford 1976). At the desired stage of development, the cells were removed from the agar surface with cold distilled water, washed by centrifugation at 1000 g for three minutes and were resuspended (4 volumes buffer/ml packed cells) in 50mM Tris-HCl (pH 7.5) containing 0.02% sodium azide 2mM mercaptoethanol and 2mM benzamidine (TAMB). Most of the preparations contained 20 gm wet weight of cells (approximately 1.5 gm protein). In some experiments the axenic strain (AX3) were used. AX3 cells were grown in HL5 media (Cocucci and Sussman 1970)

in two liter flasks on a rotary shaker. At the desired density, the cells were harvested by centrifugation in a continous flow rotor. If differentiated AX3 cells were required the amoeba were resuspended in three volumes (V/W) of 7 mM (2[N-Morpholino] ethanesulfonic Acid) pH 6.5, containing 20 mM KCl and 5 mM $MgSO_4$. Plating, and harvesting was carried out as described above. When undifferentiated AX3 amoeba were to be used, the cells were washed three times in 50mM TAMB prior to homogenization and sonication. The harvested AX3 cells routinely weighed 60-100 grams.

Cell extracts were prepared by homogenization using a Potter-Elvehjem tissue grinder (two strokes) followed by disruption by three 45 second exposures to a two cm probe of a sonic cell disrupter (Model 300, Fisher) at a setting of 45. The sonicate was centrifuged at 8,000g for thirty minutes and the supernatant obtained was centrifuged at 100,000g for 60 minutes. The 100,000g pellet was resuspended in 50mM TAMB and 20% Triton X 100 was added to give a final concentration of 0.2%. The mixture was stirred at room temperature for 20-30 min then centrifuged at 100,000g for 60 min.

2.2.3 Preparative Column Chromatography

The cytosolic 100,000g supernatant (180-400 ml) was mixed with 50 ml of DE52 cellulose that had been equilibrated in 50mM TAMB, then centrifuged at low speed to settle the resin. The supernatant was removed and the protein precipitated with ammonium sulfate (70% saturation). The ammonium sulfate pellet (15,000g for 30 min) was resuspended in 10-40ml of 50mM TAMB and dialyzed overnight against 10mM Tris- HCl (pH 7.5). The dialysate was then centrifuged at 9,200g for 15 minutes. The resulting pellet was discarded and 6-8 ml of the supernatant was applied to a Sephacryl 300 column (1.6 x 84 cm) that had been equilibrated in TAMB.

The DE52 resin was placed into a column and the cytosolic proteins that had been retained by the DE52 cellulose were eluted with an 8hr linear 0-0.2M KCl gradient. The active fractions

from the DE52 cellulose chromatography were individually pooled and concentrated by ultrafiltration (YM 10, Amicon) to 3ml, then applied to the S300 column.

Fifteen ml of the Triton solubilized proteins from the 100,000g pellet fraction were applied to a Concanavalin A (Con A) column (0.5 x 10 cm) that had been equilibrated in TAMB containing 500mM NaCl. The Con A column was washed with 50ml of the equilibration buffer followed by 50 ml of the buffer containing 0.1% Triton X 100. Finally the column was eluted with equilibration buffer containing 0.1% Triton X 100 and 1M alphamethylmannoside.

2.3.4 Preparation of ^{32}P Labeled Kemptide

Kemptide was phosphorylated using either commercial holo-enzyme or catalytic subunit of the cAMP dependent protein kinase (beef heart) or purified catalytic subunit from *D. discoideum.* The reaction mixes (approximately 1ml) contained 2 mM $MgCl_2$, 0.02 mM cAMP (only for holoenzyme), 2.5 mg/ml Kemptide and 75 uM [γ - ^{32}P]ATP (25 Ci/mMol). The reaction was allowed to proceed until most (80-90%) of the radioactivity was associated with the Kemptide, as determined by the difference between total radioactivity in a 20 µl volume and the amount of radioactivity from a duplicate volume which remained bound to a P 81 cellulose filter after passing through five 300ml acetic acid washes. The reactions were terminated by boiling for 5 minutes and the precipitated enzyme removed by centrifugation. For certain experiments the residual ATP was removed by passing the reaction mixture over Dowex 50W cation exchange resin in 200 mM glycine HCl (pH 2.5) and eluting the bound Kemptide with 200 mM Tris-HCl (pH 10). The labeled substrate was diluted prior to use with sufficient 50 mM Tris HCl (pH 7.5) to give about 100,000 cpm/25ul and brought to pH 7.5 with HCl.

2.3.5 Preparation of ^{32}P Labeled Histone

Histone VII-S, (Sigma) at a concentration of 3 mg/ml was phosphorylated using the catalytic subunit of the cAMP dependent protein kinase in reaction mixture otherwise identical to that used in labeling Kemptide. After labeling was completed the histone was then precipitated with ammonium sulfate (70% saturation) and dialyzed extensively against 50 mM TAMB to remove residual ATP. Following dialysis, either $MgCl_2$ or $MnCl_2$ was added to a concentration of 3mM.

2.3.6 Phosphatase Assays

Kemptide or histone phosphatase activity was assayed by combining 25µl of the labeled substrate with 25µl of column fractions or enzyme preparations, and incubating at 23°C for 10-120 minutes. The reactions were terminated by removing them to 1 cm square P81 cellulose filters which were then washed twice with 300 ml cold 30% acetic acid and three times with 300 ml 15% acetic acid (room temp) followed by a 300 ml acetone bath and air drying (5 min. each). Radioactivity incorporated was determined using a scintillation counter (Beckman LC50). Neutral para-nitrophenyl-phosphate (pnpp) phosphatase activity was assayed by combining 20µl of the enzyme with 100µl of a reaction mix containing 10mM pnpp and 3mM $MgCl_2$ in 50mM TAB at pH 7.5. Acid pnpp phosphatase activity was assayed by combining 20µl of the enzyme preparation with 100µlof a reaction mix containing 10mM pnpp in 50 mM sodium acetate at pH 4.0. Alkaline pnpp phosphatase was assayed by combining 20µl of the enzyme with 100µlof a reaction mix containing 10mM pnpp and 3mM $MgCl_2$ in 50mM diethanolamine pH 9. Following 10-120 min incubation at 23°C, 200µlof 1M sodium carbonate pH 10.5 was added to stop the reactions and raise the pH to 10.5. The absorbance was measured at 410nm on a spectrophotometer (Model 250, Gilford Instruments).

2.3.7 Ethanol Treatments

Fractions containing phosphatase activity were individually pooled then concentrated by ultrafiltration (YM 10, Amicon). Samples of the concentrates were then subjected to an ethanol treatment (Brandt et al. 1974; Brandt et al. 1975). To one volume of concentrate, 0.3 volumes of saturated ammonium sulfate was added, followed immediately by five volumes of room temperature ethanol. The precipitated proteins were pelleted by centrifugation, (10,000g for 30min) resuspended to one volume in 50mM TAMB (pH 7.5) and dialyzed overnight against 50mM TAMB (pH 7.5). The dialysate was centrifuged at 10,000g for 30 minutes, pelleting a fine white material which was discarded. The supernatant was reconcentrated to the original volume by ultrafiltration (YM 10, Amicon) and 3ml was loaded onto the S300 column. Samples of the soluble 100,000g fraction were also subjected to the ethanol treatment.

2.3.8 Protein kinase Assay

Protein kinase activity was assayed in a total volume of 50µl with 25µl of the enzyme sample and 25µl of a reaction mixture which contained 50 mM potassium phosphate buffer (pH 6.5), 3 mM dithiothreitol, 10 mM $MgCl_2$, 35 µM Kemptide, and 25 nM [γ-^{32}P]ATP (25Ci/mmole) either with or without 20 µM cAMP. After 15 min incubation at 25°C, the entire reaction volume was removed to 1 cm square pieces of filter paper (P81 Whatman). Papers were immediately placed in ice-cold 30% acetic acid for 5 min, were transferred through a second 5 min wash in cold 30 % acetic acid then through 3 room temp 15% acetic acid washes. The filters were placed in acetone for 5 min, then air dried and the amount of radioactivity incorporated was determined by scintillation counting.

2.4 *Results*

2.4.1 Cytological distribution and time course of development

A number of distinct Kemptide, histone and pnpp phosphatase activities are present in extracts of *Dictyostelium*. Histone and pnpp phosphatases were found in both soluble and particulate fractions, while Kemptide phosphatase was only found in the soluble fraction. When the soluble fraction was assayed for protein and pnpp phosphatase activities during development from the amoeba to the sorocarp stage, little change was observed in total soluble activities. Histone phosphatase activity in the particulate fraction decreased about 40% during development.

2.4.2 Chromatographic behavior on ion-exchange resin

When the 100,000g supernatant was applied to DE52 cellulose resin, protein and pnpp phosphatase activity was found in both the flow-through and retained fraction. Figure 2 on page 39 shows the elution of the 100,000g soluble Kemptide and histone phosphatase activities from DE52 anion exchange resin. The figure also shows the elution of neutral and acidic pnpp phosphatases that were largely separable from the protein phosphatase activities.
Kemptide phosphatase eluted as two peaks, K1 and K2, at 10mM-20mM and 150mM KCl respectively. Histone phosphatase eluted as a single broad peak from about 130mM to 180mM KCL.

The activity of K1 did not change significantly throughout the developmental cycle. The K2 activity, however, varied widely but the variation did not follow any clear developmental pattern. In general there was less K2 activity present than K1.

2.4.3 Size variations and molecular weights

as determined by gel filtration chromatography

The DE52 flow-through contained a Kemptide phosphatase that was subjected to S300 gel filtration chromatography. Figure 3 on page 40 shows the elution of the DE52 flow-through Kemptide phosphatase activity from the S300 column. The Kemptide phosphatase activity eluted from the S300 column as a single peak which just preceded and overlapped the single peak of neutral pnpp phosphatase activity. The K1 Kemptide phosphatase that was eluted from the DE52 resin in the KCl gradient was also applied to the same S300 column. The elution volume of K1 was identical to that of the flow-through activity. It therefore appeared that the flow-through and K1 activities might be catalyzed by the same enzyme. Increasing the amount of resin used by five fold, however, did not lower the percent of activity in the flow-through, indicating that overloading of the resin was not responsible for the appearance of flow-through activity.

The elution of molecular weight standards and the various soluble activities from a calibrated S300 column is shown in Figure 4 on page 41, panel C. For these molecular weight determinations, the activities were separately pooled from DE52 fractions, concentrated, and individually applied to the calibrated column. The value shown for the soluble histone phosphatase was obtained following pretreatment of the enzyme with ethanol. This procedure, as described below, was necessary because the soluble histone phosphatase eluted in a broad peak extending from near the void volume to a volume corresponding to about Mr 55K (not shown). The broadness of the peak was not due to substrate limitation as in no case was enough substrate dephosphorylated for the substrate to become rate limiting. The elution volumes and apparent molecular weights of the DE52 K1 activity, 88K, was identical to that of the flow-through activity. The K2 activity, however, eluted at a volume corresponding to an apparent molecular weight of 180K.

2.4.4 Gel filtration analysis of total 100,000g soluble phosphatase activity and evidence for subunit structures

In addition to analysis of the individual activities by S300 chromatography we have also followed the elution profiles of the phosphatases when the crude 100,000g soluble fraction was applied to the column (figure 4, panel A). While the Kemptide phosphatase activity is not resolved into two peaks there is an indication that two different sizes of activity are present. Histone phosphatase activity was observed from fraction 30 to fraction 58 indicating considerable size heterogeneity. The elution profiles of neutral and acid pnpp activities, which were largely separable from the protein phosphatase activities are also shown.

We have treated both 100,000g soluble extracts, and pooled activities from columns, with ethanol. This procedure has been widely used on mammalian phosphatases to release and purify an almost ubiquitous Mr 35K catalytic subunit from larger molecular species and to demonstrate the presence of subunits (Brandt et al. 1974; Brandt et al. 1975). Figure 4 panel B shows the elution of the ethanol treated 100,000g soluble activities from the same S300 column used in panel A. The effect is quite striking for the histone phosphatase activity. Where, prior to the treatment, the peak extended for thirty fractions, following the treatment, most of the histone phosphatase activity elutes as a very distinct peak at a volume corresponding to a molecular weight of about 55K. In most experiments this ethanol treatment resulted in 10-30% activation of the histone phosphatase. The effect of the treatment on the Kemptide phosphatase activity was to reduce the total activity by about 90% and to eliminate the higher molecular weight shoulder. The ethanol treatment also eliminated the lower molecular weight neutral pnpp activity, as shown in fig 4A and 4B and reduced the activity of the higher Mr component by 50-90%. The acid pnpp activity was reduced, but the elution pattern did not shift significantly from that of the untreated enzyme.

In addition to testing the effect of ethanol on the crude 100,000g supernatant activities, we also tested the effects of ethanol on separated histone and K2 Kemptide phosphatase activities

eluted from the DE52 resin. Figure 5 on page 42 shows the elution of untreated (panel A) and ethanol treated (panel B) DE52 K2 activity from an S300 column. The majority of the activity in panel A elutes in a peak extending from fraction 70-95 while following the treatment the activity eluted from fraction 85-110. This result tends to support the idea that the higher Mr 188K K2 activity can be converted to a lower Mr activity which is similar in size to the DE52 flow-through and K1 activities. Ethanol treatment of the lower Mr flow-through of K1 Kemptide phosphatase did not shift their elution patterns (not shown). Figure 6 on page 43 shows the elution of untreated and ethanol treated histone phosphatase from an S300 column. The shift in the elution pattern is clear and may indicate that all of the soluble histone phosphatase contains a single type of Mr 55K catalytic subunit.

2.4.5 Size relationship of the cyclic AMP-dependent protein kinase and Kemptide phosphatase

Because much of our past work has involved the cAMP dependent protein kinase (cAMP-dpk; Rutherford et al. 1982; Rutherford et al. 1984) and because cAMP-dpk activity is commonly assayed using Kemptide as phosphate acceptor, it was of interest to determine where Kemptide phosphatase eluted from a gel filtration column compared to the cAMP-dpk holoenzyme and free catalytic subunit. Figure 7 on page 44 shows the elution of 100,000g soluble Kemptide kinase and phosphatase from an S300 column. Kemptide kinase activity eluted in two peaks. The first peak, which is cAMP dependent, represents the holoenzyme and the second, which is cAMP independent, is the free catalytic subunit (Rutherford et al. 1984). The Kemptide phosphatase activity eluted in a broad peak between, and partially overlapping, the two peaks of kinase activity. It is possible that the presence of Kemptide phosphatase in the same fractions that contained cAMP dependent kinase activity caused overestimation of the Mr of the cAMP dependent holoenzyme in

some early kinase reports (Rutherford et al. 1982; Cooper et al. 1983; Leichtling et al. 1982). In support of this idea we found that the apparent peak of cAMP-dpk was shifted from fraction 27 to fraction 33, when Kinase activity was assayed in the presence of 1 mM phenanthroline, a powerful inhibitor of the Kemptide phosphatase. A second, although unrelated, observation, is that the elution of most of the phosphatase activity between the peak of cAMP dependent holoenzyme, Mr230K, (Rutherford et al. 1984) and the free catalytic subunit Mr40K (Rutherford et al. 1984) sets both upper and lower limits on the sizes of the Kemptide phosphatase activities present in the soluble fraction, and these limits are in agreement with the data presented in figure 3 panel C.

2.4.6 Phosphatase activity in the particulate fraction

In addition to assaying the 100,000g soluble fraction for different protein and pnpp phosphatase activities we also examined the 100,000g particulate material . We found that the particulate fraction contained histone phosphatase that could be solubilized by 0.2% Triton X-100. The particulate fraction did not contain significant amounts of Kemptide phosphatase. Neutral and alkaline pnpp phosphatase activities were also extracted from the particulate fraction by 0.2% Triton X-100. The high molecular weight (Mr > 300K), alkaline pnpp activity was easily separable from the neutral pnpp and histone phosphatase activities by chromatography on Concanavalin A to which it bound. The Triton solubilized histone and neutral pnpp activities appeared in the flow-through of the Con A column and co-eluted from subsequent DE52 anion exchange chromatography. These two activities were both stimulated by Mn^{++} and may reside on the same protein. An earlier report from this laboratory described the purification of an alkaline phosphatase that was bound to the plasma membrane (Armant and Rutherford 1982). The activity that is released from the 100,000g pellet shares several properties with the plasma membrane bound enzyme, such as pH optima of 9.5, binding by Con A resin, activity in the presence of 1M NaCl, inhibition by phenanthroline and reactivation following phenanthroline treatment, by Zn^{++}. It is

probable, therefore, that the alkaline pnpp phosphatase activity described here is the same as that previously described by Armant and Rutherford.

2.4.7 Inhibitors and co-factors of phosphatase activities

Phosphatase activities are often characterized not only on the basis of substrate specificity but also by their responses to various co-factors and inhibitors. Some phosphatase activities are strongly activated or inhibited by one or more divalent cations while others are unaffected. ATP can influence the activity of some phosphatases, either by covalent modification by a kinase (Hemmings et al. 1982) , through allosteric interactions (Shacter-Noiman and Chock 1983) or by chelation of essential metal ions (Hsiao et al. 1978). Table 1 on page 45 lists the phosphatase activities that we have identified in *Dictyostelium* extracts, their estimated molecular weights, (only for the soluble activities) and their responses to ATP, Mg^{++}, Mn^{++}, Pi^{2-} and phenanthroline. Several of the phosphatases were affected by phenanthroline, a compound which chelates Zn^{++}. The particulate alkaline pnpp phosphatase was inhibited by about 60% in the presence of 2mM phenanthroline while none of the other pnpp phosphatase activities were affected. Both the DE52 flow-through Kemptide phosphatase and the DE52 K1 Kemptide phosphatase were completely inhibited by 2mM phenanthroline and in both cases the activity could be restored to control levels by the addition of 2mM Zn^{2+} to the treated enzyme preparation. The DE52 K2 Kemptide phosphatase was also inhibited but only by about 35%. The inhibition of the Kemptide phosphatases by 1,10-phenanthroline and the specific recovery of their activities by the addition of Zn^{2+} suggest that they may be Zn^{2+} metaloenzymes. Significantly, none of the Kemptide phosphatases were inhibited by 10 mM EDTA or 10 mM NaF. None of the histone or Kemptide phosphatases require exogenous divalent cations for activity as all of them displayed activities at least equal to control levels in the absence of added cations. The Kemptide phosphatase activities were actually inhibited by 20 mM Mg^{++}. The influence of divalent cations on the pnpp activities was more variable. The neutral DE52 flow-through pnpp phosphatase had an absolute

requirement for divalent cations, being completely inactive without added Mg^{++} or Mn^{++}. The DE52 P1 activity was also stongly activated by added cation, giving only 30% of the control activity without cations. DE52 P2 activity, in contrast, was nearly unaffected by either Mg^{++} or Mn^{++}, giving 90% of control activity in the absence of cations. ATP at 10 mM did not activate or inhibit any of the Kemptide phosphatases while it strongly inhibited all of the neutral pnpp phosphatases. We considered the possibility that the ATP inhibition was due to chelation of Mg^{++} that is required for the DE52 flow-through neutral pnpp activity and which activates both the DE52 P1 and P2 enzymes. In fact, the inhibition of the neutral pnpp phosphatases by 10mM ATP could be relieved by adding 10 mM $MgCl_2$.

2.4.8 An artifactual inhibition of histone phosphatase

by a heat stable molecule

Several heat stable protein modifiers of protein phosphatase activities have been identified in mammalian tissues (Huang and Glinsmann 1976; Goris et al. 1978). We have examined both heated and ethanol treated *Dictyostelium* extracts for inhibitors of phosphatase activity. While no inhibition of pnpp or Kemptide activity was demonstrated, a potent inhibitor of histone phosphatase activity was present in heated extracts and in certain column fractions from ethanol treated extracts. The inhibitor is capable of reducing histone phosphatase activity by up to 90% compared to controls (not shown). The inhibitor is larger (Mr 90-120K) than mammalian protein phosphatase inhibitors which are typically in the range of Mr 15-35K (Huang and Glinsmann 1976; Goris et al. 1978; Foulkes and Cohen 1980). We found, however, that most, if not all, of the inhibition is explained by a heat stable molecule which complexes with histone and apparently limits the available substrate for the histone phosphatase. Table 2 on page 46

shows that if heated or ethanol treated extract is added to a preparation of ^{32}P-labeled histone, most of the label becomes sedimentable at low speed whereas the control label, to which only buffer was added, is not. The histone binding also occurs in non-heated 100,000g soluble preparations. The presence of this histone binding molecule can be visually determined. When samples containing the histone binding molecule are added to reaction mixtures, a cloudy white precipitate gradually becomes visible over a period of several minutes. Testing of the inhibitor with several different types of histone revealed that the effect is nonspecific and every histone tested was similarly precipitated. We also found that this histone binding molecule will inhibit the incorporation of ^{32}P-labeled histone by the cAMP dependent protein kinase. While of no apparent interest to the regulation of either the histone phosphatases or histone kinases, the occurrence of this factor in *Dictyostelium* soluble extracts should be taken into account in future investigations of inhibitors of kinase or phosphatase activity. We therefore report its presence.

2.5 *Discussion*

We have examined soluble and particulate fractions from *Dictyostelium* for phosphatase activities. Three peaks of soluble Kemptide phosphatase activity were resolved by DE52 anion exchange chromatography. One peak was located in the flow-through volume (FT), while the other two peaks K1, and K2, eluted at 10-20 mM and 150 mM KCl respectively. The FT and K1 activities elute from gel filtration at the same volume and their responses to phenanthroline are identical. In addition, they share other characteristics such as thermal stability and isoelectric point (not shown). Because of these shared characteristics, we believe that the FT and K1 activities may be nearly identical proteins. The higher molecular weight K2 activity may be related to the F.T. and K1 proteins since ethanol treatment shifts the elution of the K2 activity from gel filtration, to a volume similar to that of FT or K1. This may indicate that FT and K1 represent a catalytic activity which is complexed with additional proteins in K2.

Although for each of the three peaks of Kemptide phosphatase there is a closely associated neutral pnpp phosphatase activity, we believe that the pnpp and Kemptide activities are separable. The evidence for this is as follows. 1) While the peaks overlap on both gel filtration and anion exchange they never coincide and in some cases the peaks are 10 fractions apart. 2) None of the neutral pnpp phosphatases are inhibited by phenanthroline, whereas all of the Kemptide phosphatases are strongly inhibited by it. 3) ATP strongly inhibits the neutral pnpp phosphatase activities but does not affect any of the Kemptide phosphatases. The only evidence, beyond the similar chromatographic behavior of the activities, which supports the idea that single proteins are responsible for both types of activities, is the fact that 5 mM pnpp is a very potent inhibitor of all of the Kemptide phosphatase activities (not shown). However, it would not be surprising for pnpp to act as a competitive inhibitor rather than as an alternative substrate. Further testing should resolve this question.

In addition to soluble Kemptide phosphatase activity a soluble histone phosphatase was identified in DE52 cellulose fractions. The broad peak of histone phosphatase activity on both

anion exchange and gel filtration chromatography, is probably an indication of molecular heterogeneity. We believe that the activation and conversion to a discrete peak of activity by the ethanol treatment suggests the presence of a common 55K catalytic subunit. While the DE52 fractions containing histone phosphatase contained little neutral pnpp phosphatase activity, inspection of Figure 1 will show that a great deal of acid pnpp phosphatase activity is present. We found that the histone phosphatase activity present in pooled DE52 fractions was insensitive to changes in pH between 4 and 7.5 (not shown). It therefore, may be, that at the extremes of pH, two different enzymes, one a neutral histone phosphatase and the other an acid phosphatase are able to dephosphorylate the histone substrate. On the other hand it may also be that the histone phosphatase is simply able to tolerate a remarkably wide pH range.

Although at least two low molecular weight, heat stable protein phosphatase regulatory proteins have been identified in mammalian tissues, we found no such modulators in heated *Dictyostelium* extracts. The only significant inhibitory component in heated or ethanol treated extracts appears to be associated with a high molecular weight protein which complexes with histone, preventing dephosphorylation by the histone phosphatase.

We have begun, and are continuing, an effort to identify the molecular components which regulate cAMP effects intracellularly in *Dictyostelium.* We have previously described a cAMP dependent protein kinase which is believed to be the primary intracellular mediator of cAMP effects in other systems (Rutherford et al. 1982; Rutherford et al. 1974). The present report, represents the first detailed description of protein phosphatase activities present in *Dicyostelium.* These protein phosphatase activities are capable of dephosphorylating substrates which had previously been phosphorylated by the cAMP-dpk and may therefore represent a significant and previously unreported part of the cAMP dependent, regulatory mechanisms in *Dictyostelium.* In addition to their possible *in vivo* significance to cAMP regulated events, a knowledge of the protein phosphatase activities that are present in *Dictyostelium* is potentially important to any investigation of protein kinases. For example, the presence of a functional phosphatase may mask the activity of the kinase. In addition, the overlapping of a kinase and a phosphatase in gel filtration fractions could lead to misinterpretations of molecular weights.

While the five protein phosphatase activities described here have clearly and reproducibly been resolved from each other it remains questionable how many of these are related proteins. We have demonstrated that treatment with ethanol causes a shift in the elution pattern of both histone and Kemptide phosphatase activities. It is possible that this decrease in the apparent molecular weights of these proteins is a result of the loss of regulatory components. It may be that all of the Kemptide phosphatase activities are related proteins and that all the histone phosphatase activities contain an identical catalytic component. Further investigation will resolve the question of the relationships of these proteins to each other and to the cAMP dependent protein kinase.

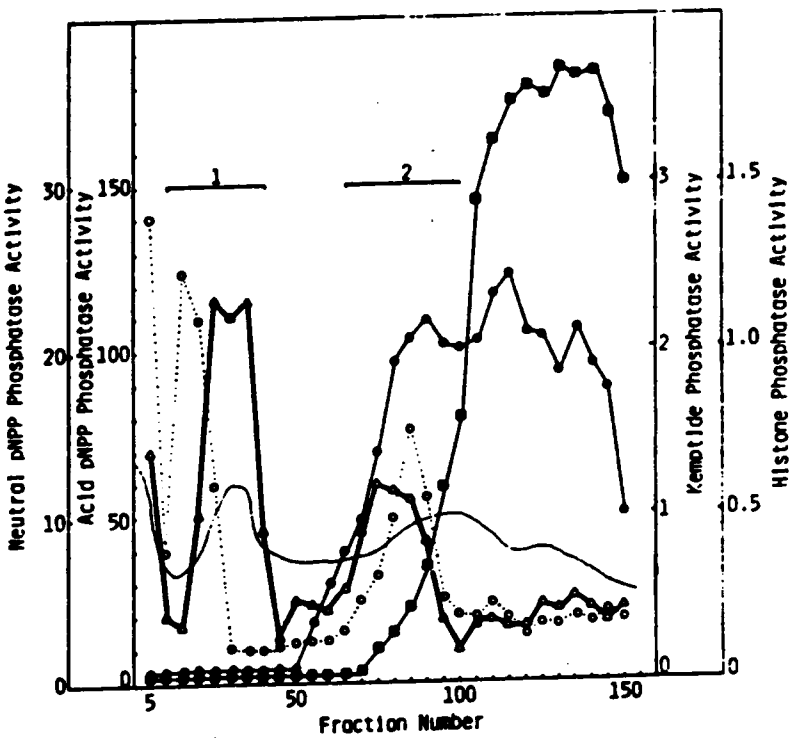

Figure 2. **DE52 Chromatography:** The column was loaded with the resin containing the bound proteins and washed with 50mM TAMB (pH7.5) pumped at 160ml/hr; 4 min fractions were collected. After the absorbance (shown by the continuous thin curving line) as determined by a column monitor had returned to baseline, an 8hr linear 0-0.2 M KCl gradient was started. •—• Histone phosphatase activity. O—O Kemptide phosphatase activity. ▪—▪ Acid pnpp phosphatase activity. Δ —Δ Neutral pnpp phosphatase activity. 1 Fractions containing K1 and P1 activity. 2 Fractions containing K2 and P2 activity. (—) A280 For the protein substrates, one unit of activity is defined as 1 picomole of phosphate released/min/ml. For pnpp, one unit of activity is defined as 1 micromole of phosphate released/min/ml.

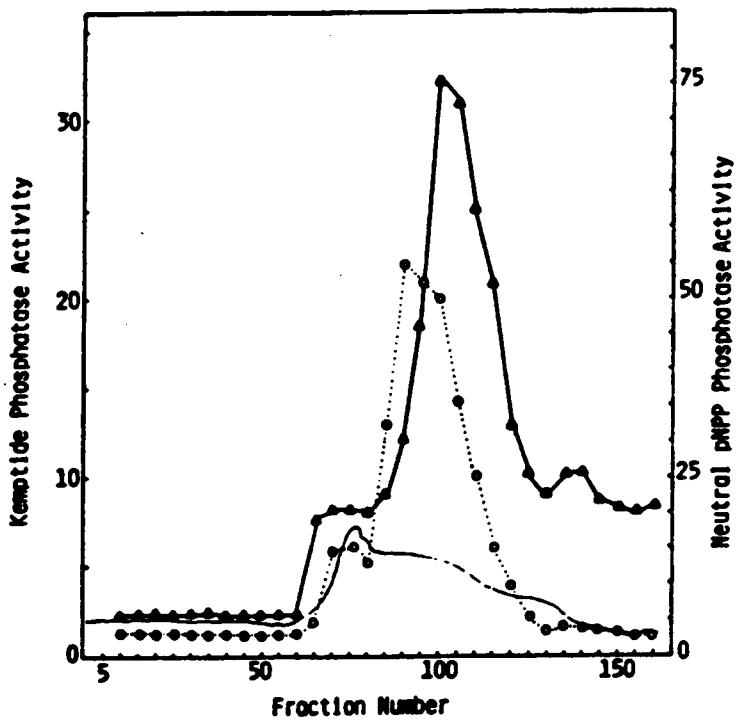

Figure 3. Sephacryl 300 chromatography: The DE52 flow-through volume was concentrated and 6-8 ml loaded onto the S300 column (1.6 x 84cm). One ml fractions were collected. O—O Kemptide phosphatase activity.▲ —▲Neutral pnpp activity.(—) A280 One unit of activity is defined as 1 picomole/min/ml for both substrates.

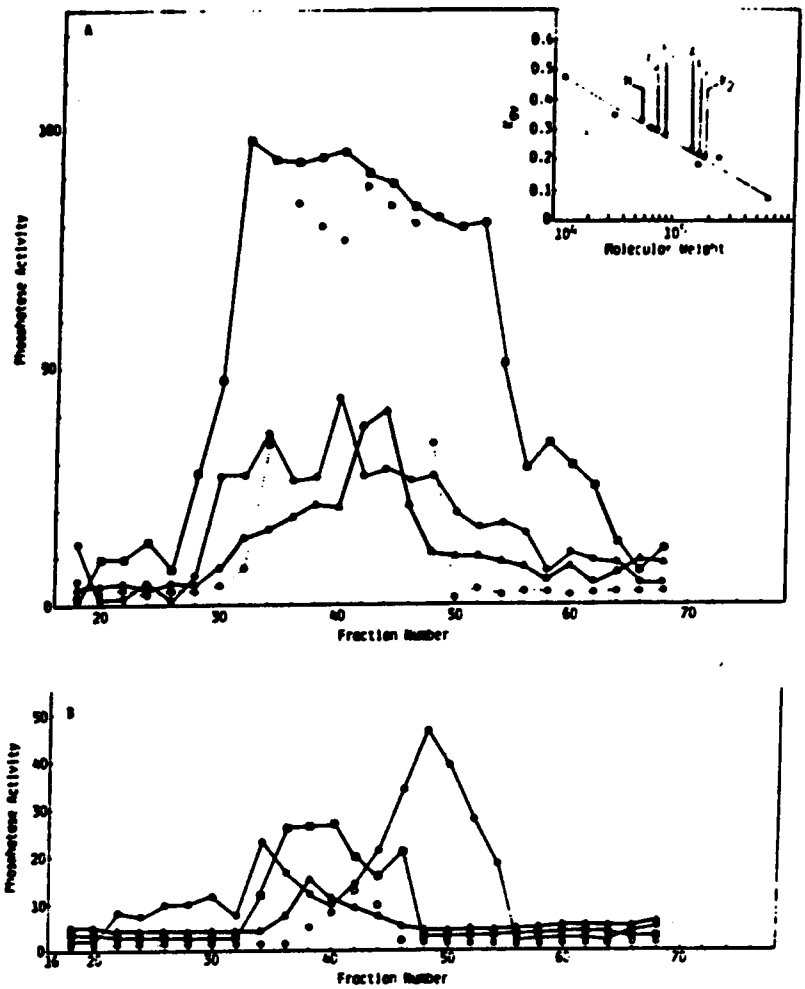

Figure 4. **Molecular weights and Gel filtration analysis of total 100,000 g soluble activities before and after ethanol treatment:** Panel C Standards (•) were used to calibrate an S300 column. The individual activities from pooled DE52 or S300 fractions were separately applied to the calibrated column. H shows the Mr apparent of ethanol treated soluble histone phosphatase activity. P_1 shows the Mr apparent of the peak 1 and DE52 flow-through pnpp activities. K_1 shows the Mr apparent of the peak 1 and DE52 flow-through Kemptide activities. A shows the Mr apparent of the acid pnpp activity. P_2 shows the Mr apparent of the peak 2 pnpp activity K_2 shows the Mr apparent of the peak 2 Kemptide activity. Panel A. Six ml of the crude 100,000 g soluble fraction was applied to an S300 column. Four minute fractions of 2.5 ml were collected. •—• Histone phosphatase One unit = 1 picomole PO_4 released/min/ml O—O Kemptide phosphatase One unit = 1 picomole PO_4 released/min/ml Δ—Δ Neutral pnpp phosphatase One unit = 1 micromole PO_4 released/min/ml ▪—▪ Acid pnpp phosphatase One unit = 1 micromole PO_4 released/min/ml Panel B. Six ml of the 100,000 g soluble fraction was subjected to an ethanol treatment. Following dialysis and reconcentration the six ml was applied to the same column used in panel A. Four minute fractions of 2.5 ml were collected. Symbols and units of activity are as in panel A.

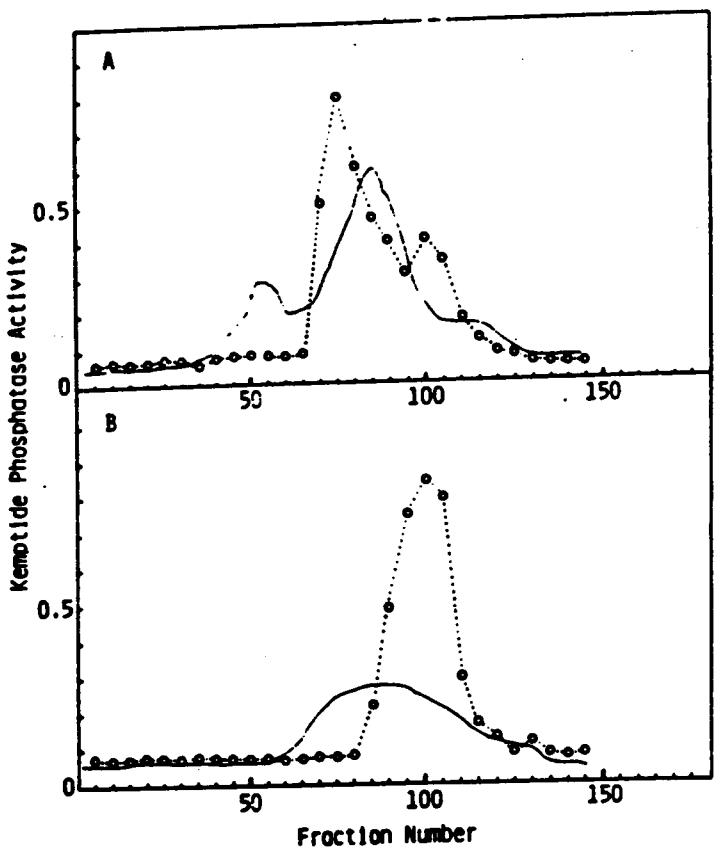

Figure 5. **Ethanol shifted Kemptide phosphatase:** Panel A shows the elution of untreated peak 2 Kemptide phosphatase activity from the S300 column. Panel B shows the elution of the ethanol treated activity from the same column. Panels A and B, one ml fractions were collected.(O-O) Kemptide phosphatase. (—) A280
One unit of activity = 1 picomole PO_4 released/min/ml.

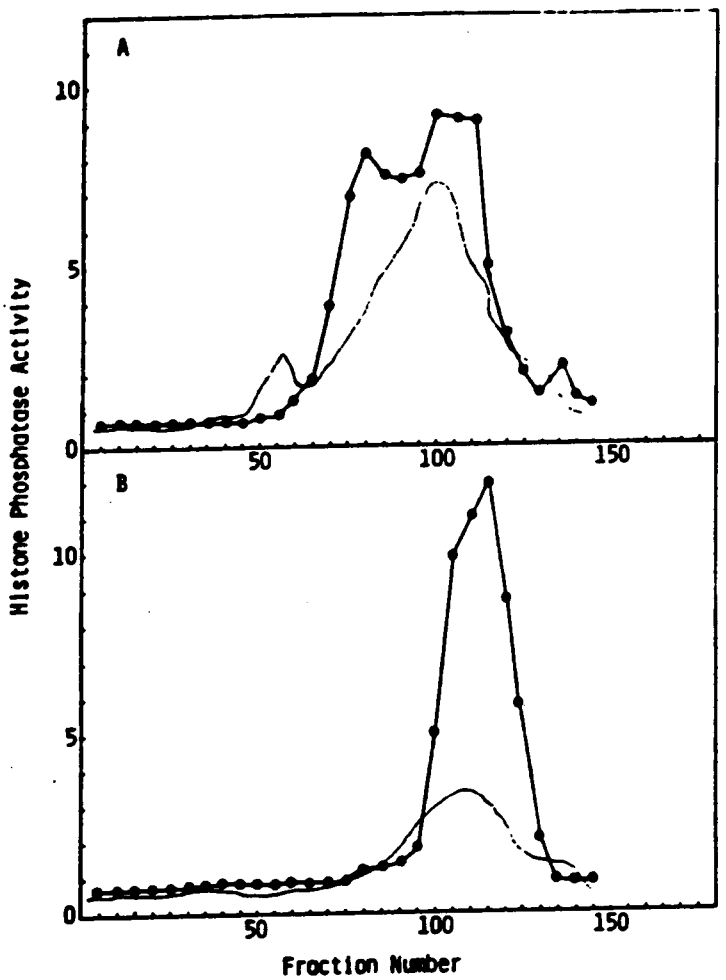

Figure 6. Ethanol shifted histone phosphatase: Panel A shows the elution of the untreated histone phosphatase activity from the S300 column. Panel B shows the elution of the treated histone phosphatase activity from the same column. Panels A and B, one ml fractions were collected. (O-O) Histone phosphatase. (—) A280 One unit of activity = 1 picomole PO_4 released/min/ml.

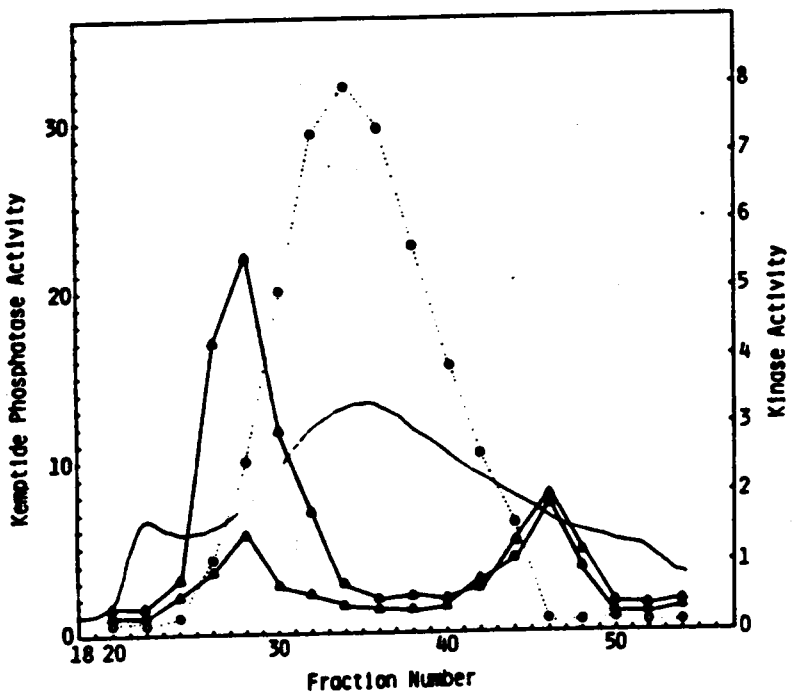

Figure 7. **S300 chromatography of Kemptide phosphatase and cAMP dependent protein kinase:** A 3 ml sample of the crude 100,000 g soluble fraction was applied to the column. 2.5 ml fractions were collected. ▲ Kinase activity in the presence of cAMP. Δ Kinase activity in the absence of cAMP. *O* Kemptide phosphatase activity. Kemptide phosphatase, one unit = 1 picomole PO_4 released/min/ml. One unit of kinase activity = 1 picomole PO_4 incorporated/min/ml.

Table 1. The effects of inhibitors and cofactors

Source	Enzyme	M_r	Percent control[a] activity in the presence of							
			2 mM Phenanthroline	No cation	5 mM $MgCl_2$	20 mM $MgCl_2$	10 mM ATP	10 mM NaF	15 mM PO_4	5 mM $MnCl_2$
Soluble	DE52 FT Kemptide	97K	0	104	101	71	105	121	96	111
Soluble	DE52 neutral pNPP	78K	0	96	96	82	99	103	88	91
Soluble	DE52 P_1 Kemptide	97K	65	112	102	69	111	97	92	106
Soluble	DE52 P_2 neutral pNPP	78K	100	110	92	40	96	93	81	120
Soluble	DE52 P_2 Kemptide	180K	102	2	96	68	48	62	82	123
Soluble	DE52 P_3 neutral pNPP	170K	99	30	103	84	62	51	85	101
Soluble	DE52 acid pNPP	150K	105	90	106	100	54	50	89	104
Soluble	Histone	55K	98	98	100	95	97	102	91	108
Particulate	Histone	—	101	115	103	NT[b]	96	98	75	125
Particulate	Alkaline pNPP	—	40	80	96	NT	NT	NT	NT	70
Particulate	Neutral pNPP	—	104	0	103	NT	NT	NT	NT	800

[a] Control activity, the activity of the enzyme using the appropriate buffer and substrate in the presence of 3 mM $MgCl_2$.
[b] NT, not tested.

Table 2. A heat-stable, histone binding protein

Table 2. Precipitation of Histone by a Heat Stable Protein

Addition to ^{32}P-Histone	Percent of Total Radioactivity Recovered	
	Supernatant	Pellet
Heated Extract	28	72
Ethanol Treated Extract	13	87
Untreated Extract	23	77
Buffer	100	0

3. Paranitrophenol phosphatases in Dictyostelium discoideum

Dictyostelium discoideum is frequently used as a model for eucaryotic development. The life cycle of this organism includes a unicellular amoeboid stage followed by aggregation of thousands of amoeba to form a multicellular individual. The cells comprising the aggregate perform a series of morphogenic movements and eventually form a small fruiting body consisting of a slender cellulosic stalk supporting a small spore head. Because aggregation and multicelluar development in this organism both require cyclic adenosine monophosphate, we have been interested in characterizing protein phosphatases that might regulate the same substrates as the cAMP-dependent protein kinase (Rutherford et al. 1982). During the course of our work on protein phosphatases (Ferris & Rutherford 1986) we also found a number of p-nitrophenyl phosphate (pnpp) phosphatase activities. Here we report on the resolution and characterization of the pnpp phosphatase activities that were found in developing *Dictyostelium discoideum.*

Although these activities are described as pnpp phosphatases, pnpp is obviously not a physiological substrate for any enzyme and we are not predicting what the actual in vivo substrates are for any of these activities. It should be noted however, that it is not unusual for phosphatases

to dephosphorylate both proteins and pnpp (Weller 1979). Recently, it has been suggested that the physiological function of acid and alkaline phosphatases, enzymes that have traditionally been regarded as having little to do with protein substrates, is to dephosphorylate a variety of proteins involved in growth regulation. Swarup et al.(1981) reported that alkaline phosphatase from mammalian tissues possesses tyrosine specific protein phosphatase activity. As was found with the alkaline phosphatase, tyrosine phosphatase activity is also associated with acid phosphatase from human tissues (Heng-Chun et al. 1984). Given the widespread occurrence of phosphatases capable of dephosphorylating both pnpp and phosphoproteins, it is possible that some of the activities described here function in vivo as protein phosphatases and that they were simply not tested with the appropriate phosphoproteins. On the other hand, their physiological functions may be limited to hydrolyzing small phosphomonoesters.

Paranitrophenol phosphate phosphatase activities have been previously reported in Dictyostelium. Armant and Rutherford (1981) purified a plasma membrane alkaline phosphatase to apparent homogeneity and demonstrated that it also possessed 5'nucleotidase activity. They reported that the alkaline phosphatase required zinc as co-factor and that the enzyme was localized to the interface between prestalk and prespore cells in culminating Dictyostelium (Armant et al. 1980; Armant & Rutherford 1981). Two isozymes of acid phosphatase have also been found in Dictyostelium (Armant & Rutherford 1979; Oohata 1983). These enzymes were found to be prestalk specific and have been used as stalk-cell markers.

3.1 Materials and Methods

3.1.1 Materials

DE52 was from Whatman, Sephacryl S300 from Pharmacia, Sephadex G100 and all other reagents were from Sigma.

3.1.2 Harvesting of Cells

Growth and differentiation of *D. discoideum* (NC 4) was carried out as previously described (Rutherford 1976). At the desired stage of development the cells were removed from the agar surface with cold distilled water, washed by centrifugation at 1000 g for three minutes and were resuspended (4 volumes buffer/ml packed cells) in 50mM Tris-HCl (pH 7.5) containing 0.02% sodium azide 2mM mercaptoethanol and 2mM benzamidine (TAMB). Most of the preparations contained 20 gm wet weight of cells (approximately 1.5 gm protein). In some experiments the axenic strain (AX3) were used. AX3 cells were grown in HL5 (Cocucci & Sussman 1970) media in two liter flasks on a rotary shaker. At the desired density the cells were harvested by centrifugation in a continuous flow rotor. If differentiated AX3 cells were required the amoeba were resuspended in three volumes (V/W) of 7 mM (2[N-Morpholino] ethanesulfonic Acid) pH 6.5, containing 20 mM KCl and 5 mM $MgSO_4$. Plating, and harvesting was carried out as described above. When undifferentiated AX3 amoeba were to be used, the cells were washed three times in 50mM TAMB prior to homogenization and sonication. The harvested AX3 cells routinely weighed 60-100 grams.

3.1.3 Preparation of cell extracts

Cell extracts were prepared by homogenization using a Potter-Elvehjem tissue grinder (two strokes) followed by disruption by three 45 second exposures to a two cm probe of a sonic cell disrupter (Model 300, Fisher) at a setting of 45. The sonicate was centrifuged at 8,000g for thirty minutes and the supernatant obtained was centrifuged at 100,000g for 60 minutes. The 100,000g pellet was resuspended in 50mM TAMB and 20% Triton X 100 was added to give a final concentration of 0.2%. The mixture was stirred at room temperature for 20-30 min then centrifuged at 100,000g for 60 min.

3.1.4 Preparative Column Chromatography

The cytosolic 100,000g supernatant, (180-400 ml) was mixed with 50 ml of DE52 cellulose that had been equilibrated in 50mM TAMB, then centrifuged at low speed to settle the resin. The supernatant was removed and the protein precipitated with ammonium sulfate (70% saturation). The ammonium sulfate pellet (15,000g for 30 min) was resuspended in 10-40ml of 50mM TAMB and dialyzed overnight against 10mM Tris- HCl (pH 7.5). The dialysate was then centrifuged at 9,200g for 15 minutes. The resulting pellet was discarded and 6-8 ml of the supernatant was applied to a Sephacryl 300 column (1.6 x 84 cm) that had been equilibrated in 50mM TAMB.

The DE52 resin was placed into a column and the cytosolic proteins that had been retained by the DE52 cellulose were eluted with an 8hr linear 0-0.2M KCl gradient. The active fractions from the DE52 cellulose chromatography were individually pooled and concentrated by ultrafiltration (YM 10, Amicon) to 3ml, then applied to the S300 column.

Fifteen ml of the Triton solubilized proteins from the 100,000g pellet fraction were applied to a Concanavalin A (Con A) column (0.5 x 10 cm) that had been equilibrated in 50mM TAMB containing 500mM NaCl. The Con A column was washed with 50ml of the equilibration buffer

followed by 50 ml of the buffer containing 0.1% Triton X 100. Finally the column was eluted with equilibration buffer containing 0.1% Triton X 100 and 1M alphamethylmannoside.

3.1.5 Phosphatase assays

Neutral pnpp phosphatase was assayed combining 20µl of the enzyme with 100µl of a reaction mix containing 10mM pnpp and 3mM $MgCl_2$ in 50mM TAB at pH 7.5. Acid pnpp phosphatase activity was assayed by combining 20 µl of the enzyme preparation with 100 µlof a reaction mix containing 10mM pnpp in 50 mM sodium acetate at pH 4.0. Alkaline pnpp phosphatase was assayed by combining 20 µl of the enzyme with 100 µlof a reaction mix containing 10mM pnpp and 3mM $MgCl_2$ in 50mM diethanolamine pH 9. Following 10-120 min incubation at 23°C, 200 µlof 1M sodium carbonate pH 10.5 was added to stop the reactions and raise the pH to 10.5. The absorbance was measured at 410 nm on a spectrophotometer (Model 250, Gilford Instruments).

3.1.6 Ethanol Treatment of DE170

Active fractions of DE170 from a DE52 gradient were pooled and concentrated to 4.5 ml by ultrafiltration (YM 10, Amicon). Four ml of the concentrate was subjected to an ethanol treatment (Brandt et al. 1974,1975) to determine subunit structure. Briefly, 0.5ml of saturated ammonium sulfate solution was added to the enzyme, followed by 5 volumes of room temperature ethanol. The precipitated protein was pelleted by centrifugation, (10,000g for 30min) resuspended in three ml of 50mM TAMB (pH 7.5) and dialyzed overnight against 50mM TAMB (pH 7.5). The dialysate was centrifuged at 10,000g for 30 minutes, pelleting a fine white material which was discarded. The supernatant was concentrated to 0.5 ml by ultrafiltration (YM 10, Amicon) and loaded onto a small G-100 column (0.8 x 14.5cm).

3.2 *Results*

3.2.1 Resolution of Four Soluble Phosphatase Activites

The 100,000g supernatant was loaded onto a DE52 column and the flow through volume was concentrated by 70% solid ammonium sulfate precipitation. Following dialysis, the concentrate was applied to an S300 column. Two peaks of PNPP phosphatase activity were resolved by S300 chromatography (Figure 8 on page 54). The first minor peak, (S.v) eluted in the void volume of the column and was very unstable. Often, within hours of running the column the activity become virtually undetectable. No attempt was made to characterize this activity. The second peak (S70) which eluted at about 100 ml was very active and remarkably stable. It could usually be stored at 4 degrees for a week without significant loss of activity. The material which bound to the DE52 resin was eluted with an 8hr 0-0.2M KCl gradient. Three PNPP phosphatase activities were resolved. The major neutral activity (DE70) eluted at about 25 mM KCl while the second neutral activity (DE170) eluted at 100-130 mM KCl. A broad peak of acid pnpp phosphatase eluted just after DE170 (Figure 9 on page 55).

3.2.2 Particulate Protein Phosphatase Activity

The 100,000g pellet contained two pnpp phosphatases designated GP 1 and GP 2, which were separated by DE52 anion exchange chromatography (Figure 10 on page 56). The flow-through volume contained GP 1 activity, while GP 2 eluted from the resin at about 100 mM KCl. The GP1 activity in the DE52 flow-through could still be pelleted by centrifugation at 100,000g, however, following treatment with 0.1% Triton X 100, the activity remained in the high speed supernatant. GP1 was further purified by concanavalin A (Con A) affinity chromatography. After

washing the column with salt and triton, the enzyme was specifically eluted with alpha-methylmannoside.

3.2.3 Molecular Weight Determinations

Molecular weight estimates for the soluble enzymes were obtained using a calibrated Sephacryl S300 column Both S70 and DE70 elute from the column at a volume corresponding to an apparent molecular weight of about 70,000 daltons. DE170 elutes at a volume corresponding to an apparent molecular weight of 170,000 daltons. The acid phosphatase had an apparent molecular weight of 150,000 daltons. Triton treated GP 1 eluted in the void volume indicating an apparent molecular weight in excess of 300,000 daltons. S70, DE70 and GP 2 were separately applied to a calibrated Sephadex G-100 column and the results confirmed apparent molecular weights near 70,000 daltons for S70 and DE70, but GP2 eluted in the void volume, indicating an apparent molecular weight in excess of 100,000 daltons.

3.2.4 pH Optima and Divalent Cation Activation

Both DE70 and S70 had optima near pH 7.5 (Figure 11 on page 58). DE170 displayed an optimum at about pH 6.5. GP1 in marked contrast, had an optimum at pH 9.5 and the acid phosphatase pH optimum was at 4.0. Prior to determining divalent cation requirements, S70, DE70 and DE170 were concentrated and separately applied to Sephadex G100 columns to remove any unbound cations. The resulting peak fractions were tested with PNPP reaction mixes containing various concentrations of $MgCl_2$ or $MnCl_2$ (Figure 12 on page 59). As shown in the figure, S70 had an absolute requirement for divalent cation, being completely inactive without added Mg^{2+} or Mn^{2+} but the concentrations required were only in the micromolar range. Maximal activation of S70 by Mg^{2+} was higher than with Mn^{2+} and S70 was inhibited by levels

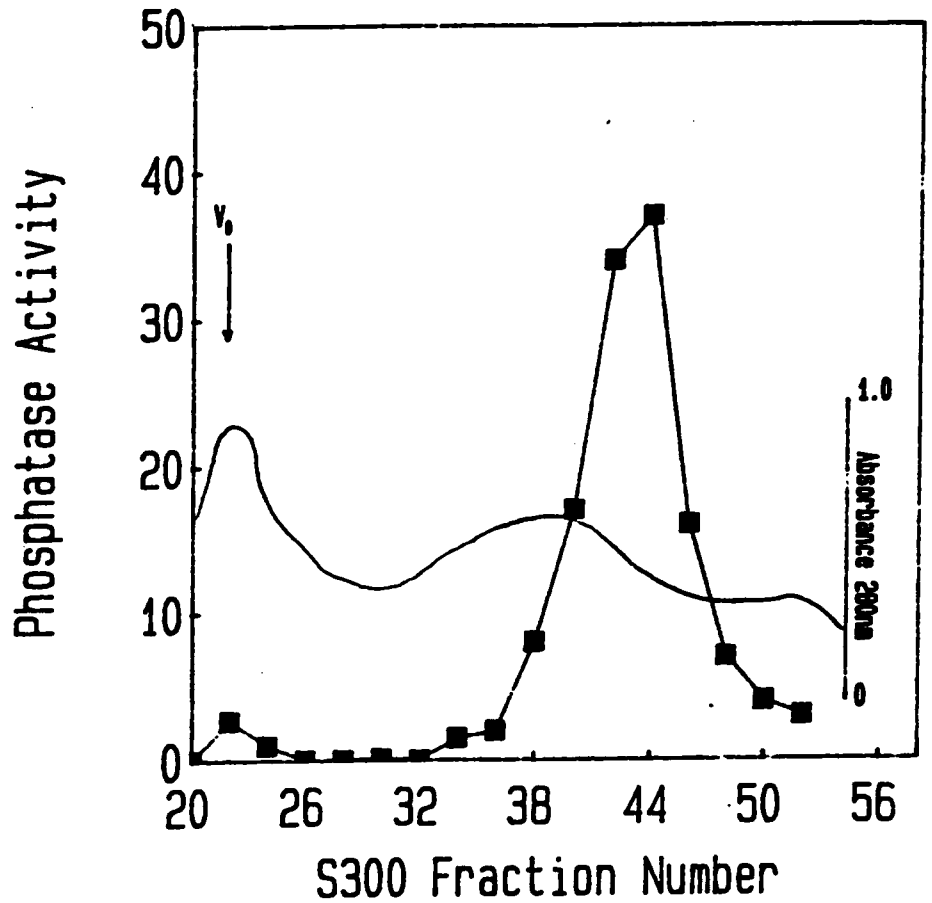

Figure 8. **S300 chromatography:** Two ml of the concentrated DE52 flow-through was eluted from the S300 column. (■) neutral pnpp phosphatase activity, micromoles Pi released/min/ml. The thin continuous line represents absorbance at 280nm.

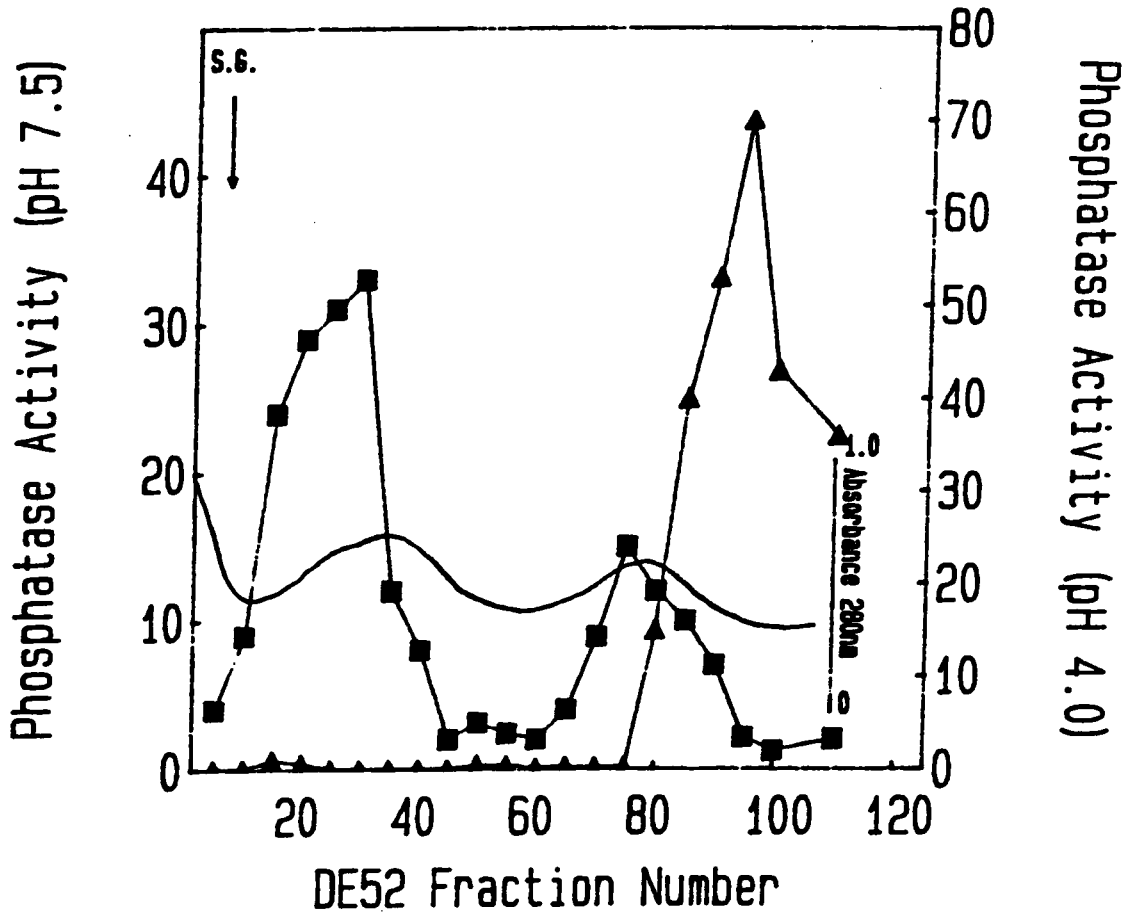

Figure 9. **Soluble DE52 pnpp phosphatase activity:** The soluble extract was applied to the resin and eluted with an 8hr linear 0-200 mM KCl gradient. (■) neutral pnpp phosphatase. (▲) acid pnpp phosphatase. Activity is expressed as micromoles Pi released/min.ml. (—) Absorbance at 280nm.

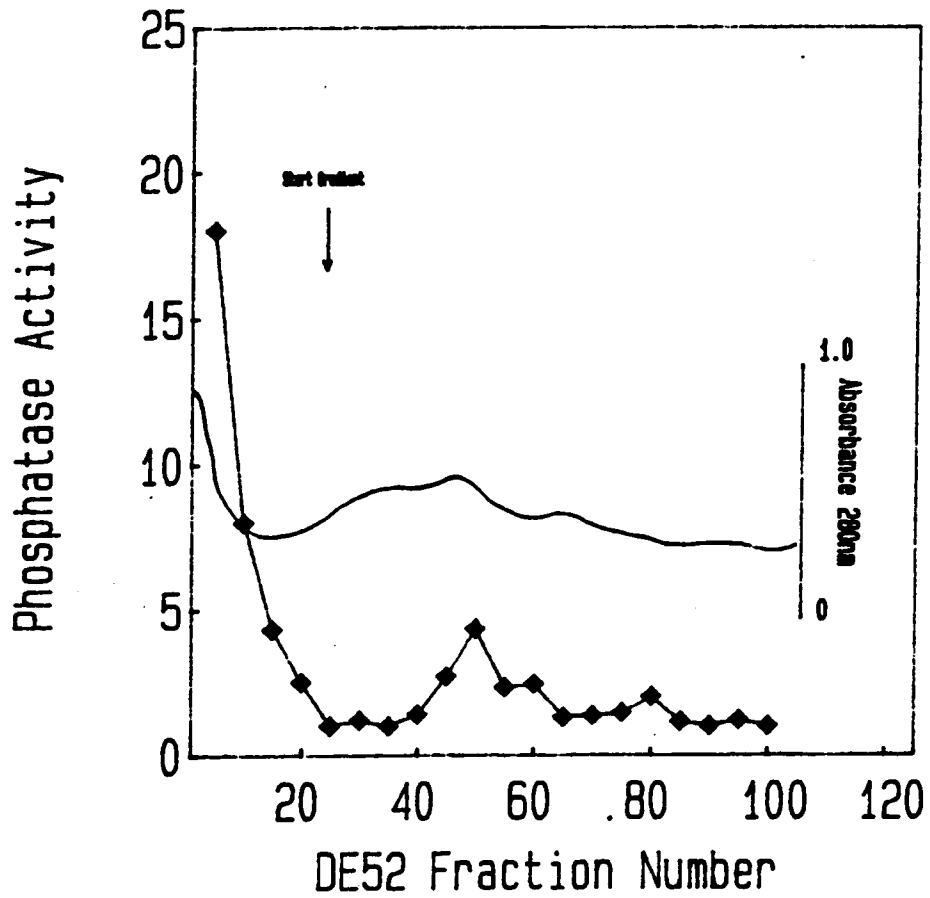

Figure 10. **Particulate pnpp phosphatase activity:** The 100,000g pellet was resuspended in buffer and applied to the resin. The column was eluted with an 8hr linear 0-200mM KCl gradient. (▪) Phosphatase activity, micromoles Pi released/min/ml. The thin continuous line represents absorbance at 280nm.

of Mg^{2+} higher than about 1.6 mM. DE70 was less dependent on added cations and retained some activity with no added cations even in the presence of 10 mM EDTA. The K 0.5 for both of the cations was in the 30-40 uM range. As shown in the figure, the two cations were equally effective in activating DE80. In contrast to S70 and DE70, DE170 displayed very little dependence on added cations, nor did higher concentrations cause the inhibitory effects seen with S70. DE170 was somewhat more active in the presence of Mn^{2+} than Mg^{2+}. Levels of EDTA up to 20mM had no inhibitory effect on the basal activity of DE170 in the absence of added cations and even caused slight activation. The K 0.5s for the acid phosphatase and for the particulate activities, GP 1 and GP 2, were not determined, however all three enzymes were active with PNPP without added cations and in the presence of 10mM EDTA.

3.2.5 Inhibitors

Table 3 on page 62 shows the effects of ATP, Pi, and NaF on the three neutral phosphatase activities. DE70, DE170, and S70 were all inhibited by 25mM NaF. The pnpp activity of DE70 in the absence of exogenous divalent cation was inhibited by about 90% and this inhibition was not effected by the addition of 8mM $MgCl_2$. The activity of DE170 in the absence of added cation was inhibited by about 70% but with 8mM $MgCl_2$ added the inhibition was only about 40%. S70 activity in the presence of 8mM $MgCl_2$ was inhibited about 60% by 25mM NaF. The effect of ATP on enzyme activity was determined using PNPP reaction mixture containing 2.5 mM $MgCl_2$ and various concentrations of ATP. All three soluble neutral enzymes (S70, DE70, and DE170) were significantly inhibited by 5 mM ATP. In subsequent experiments with DE170 we have found that the inhibitory effect of ATP can be overcome by increasing $MgCl_2$ concentrations.

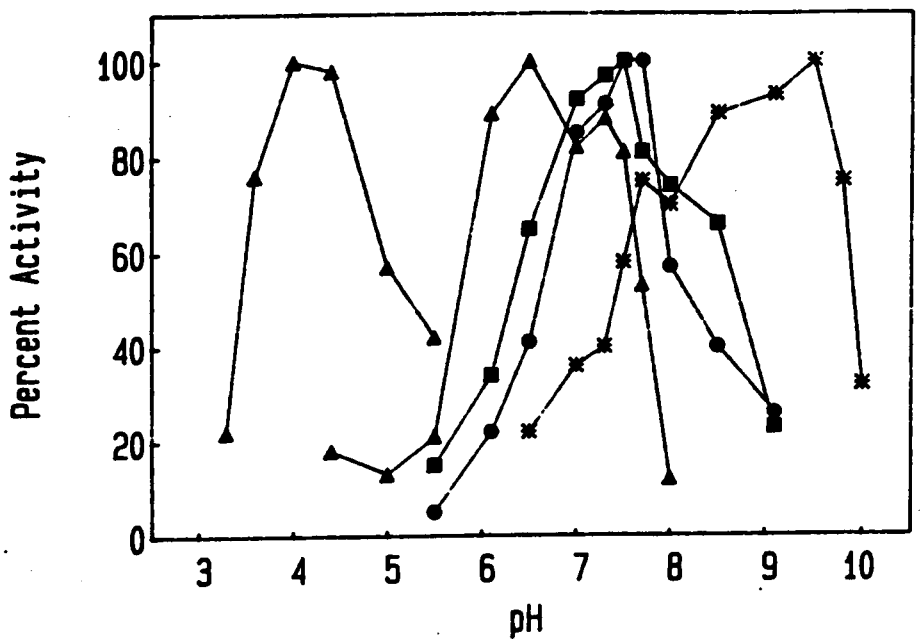

Figure 11. pH optima for pnpp phosphatases: (■) S70, (●) DE70, (▲) DE170, (▲) acid phosphatase, (✱) GP1. The enzymes were assayed in appropriate buffers in the presence of 5mM $MgCl_2$.

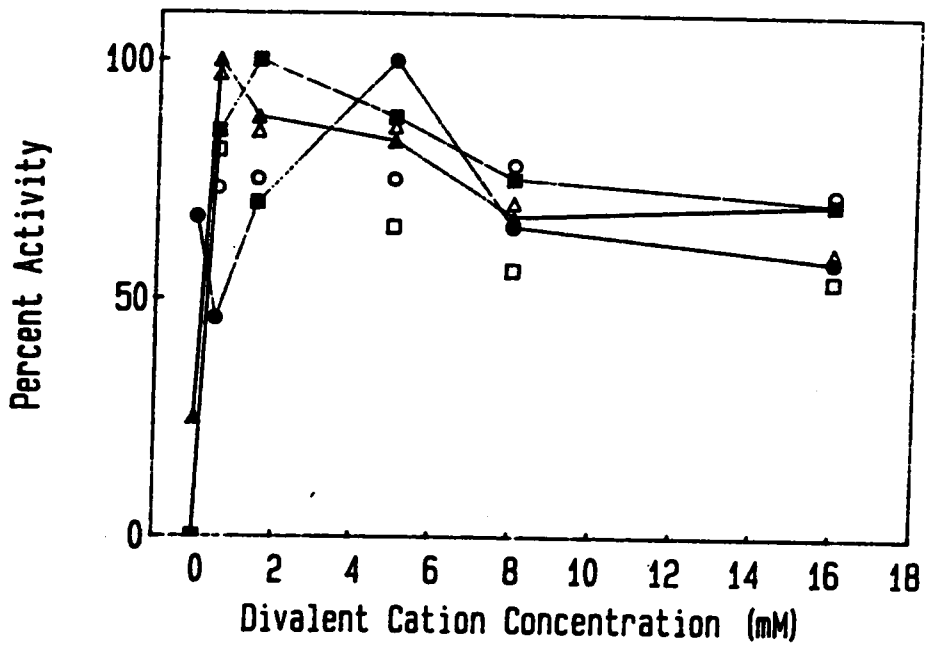

Figure 12. **Cation activation of neutral activities:** closed and open symbols represent activities with Mg^{2+} and Mn^{2+} respectively. (■) S70, (▲) DE70, (●) DE170.

3.2.6 Evidence for Subunit Structure of DE170

We have examined DE70, S70 and DE170 for subunit structure using freeze thawing in the presence of 30mM mercaptoethanol and by ethanol treatment (Brandt et al. 1975). The freeze thawing with mercaptoethanol had no effect on the activity or elution of S70, DE70 or DE170 from gel filtration. The alcohol treatment resulted in the total loss of S70 and DE70 phosphatase activities. Alcohol treatment reduced DE170 activity by about 90%. Figure 13 on page 61 shows the elution volumes of the treated and nontreated DE170 PNPP activity from a small (0.8 x 14.5cm) Sephadex G100 column. The shift in elution volume indicates a substantial reduction in the size of the treated activity.

3.3 Discussion

The inhibition of DE70, S70 and DE170 activity by ATP may be an indication that all three enzymes contain allosteric binding sites for ATP. However, the activity of some phosphatases is modulated by phosphorylation and it is possible that the enzyme preparations used contained kinase activity. An alternative explanation for the inhibition is that an ATPase is liberating inorganic phosphate, a known inhibitor of these enzymes. We have tested for ATP loss in the DE170 preparation using a spectrophotometric linked enzyme assay and found that in two hours under the same conditions used to measure ATP inhibition, 20mM ATP was reduced to 15mM. The level of inhibition caused by 20 mM ATP (nearly 100%) is considerably greater than that caused by 15mM $PO4^{2-}$, i.e. the maximum amount of phosphate that could have been produced by the amount of ATP degraded. Additionally the observation that 8mM $MgCl_2$ relieves the inhibition of DE170 caused by 10 mM ATP, while it does not relieve the inhibition caused by 30 mM $PO4^{2-}$, argues against $PO4^{2-}$ liberation as a cause of ATP inhibition. Several of the

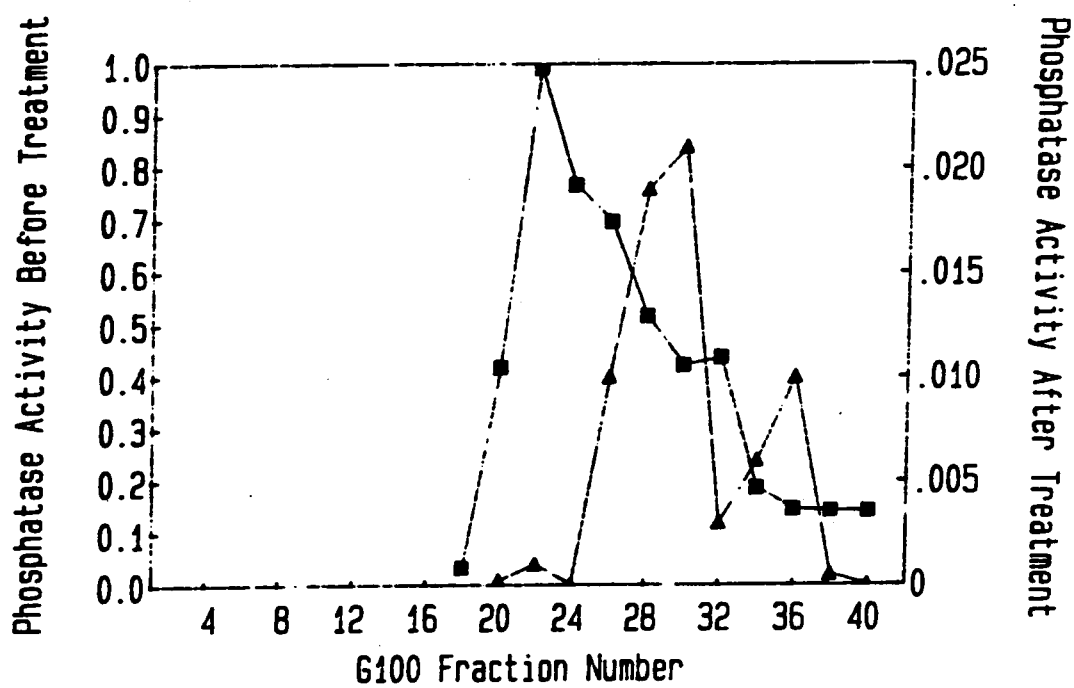

Figure 13. **Subunit structure of DE170:** One half ml samples of DE170 before and after ethanol treatment, were applied to the G100 gel filtration column. (■) before treatment. (▲) after treatment.

Table 3. The effects of ATP, Pi and NaF on pnpp phosphatases

	% Activity in the presence of						
	2mM Mg	No Cation	25mM NaF	25mM NaF+8mM Mg	8mM Mg+24mM PO_4	2mM Mg+8mM ATP	8mM Mg
S70	100	8	8	38	37	24	72
DE70	100	88	6	10	46	55	98
DE170	100	99	28	58	74	25	102

characteristics of GP1 suggest that it is the same alkaline phosphatase previously purified by Armant and Rutherford (1982). The characteristics which the activities share are as follows: 1. Membrane bound, extracted with triton. 2. Alkaline pH optimum 3. Inhibited by phenanthroline 4. Recovery of activity in presence of phenanthroline by Zn addition. 5. Binding to Con A and specific elution by alpha methylmannoside. While purification of GP1 and bona fide alkaline phosphatase would have resolved the question of their relatedness that was not done because following the Con A step, GP1 had no apparent protein phosphatase activity in our assays and at the time our interest was in pursuing phosphatases which might antagonize the actions of the cAMP-dependent protein kinase.

In light of the finding that both acid and alkaline pnpp phosphatases from mammalian tissues can dephosphorylate phophotyrosine at neutral pH, the investigation of these activities in Dictyostelium would be of great interest. Li and co-workers have suggested that the real physiological function of these acid and alkaline phosphatase is to regulate proteins phosphorylated on tyrosine residues (1982). While to my knowledge no one has yet demonstrated the presence of phosphotyrosine in Dictyostelium it has been reported to occur in yeast and it probably is present in *Dictyostelium* as well. In *Dictyostelium* both acid and alkaline phosphatase are developmentally regulated and both are localized to prestalk type cells. Intracellularly alkaline phosphatase is found tightly associated with the plasma membrane (Armant and Rutherford 1982) as are most of the tyrosine kinases and tyrosine kinase substrates that have been identified in mammals (Nigg et al. 1983; Glenney 1985). The membrane localization of alkaline phosphatase and tyrosine kinase substrates may be merely coincidental but it is suggestive, and certainly worthy of close inspection. It is worth repeating that neutral pnpp hydrolyzing activity has also quite often been found associated with purified preparations of protein phosphatases. Thus the fact that the neutral pnpp phosphatases described here were dissociable from the majority of the histone and Kemptide phosphatase activities does not indicate that small phosphomonoesters rather than proteins are the actual substrates of the pnpp phosphatases.

4. Evidence for protein kinase C in Dictyostelium

4.1 Introduction

The role of calcium as an important second messenger for a variety of extracellular stimuli, is widely accepted. It is now clear that two general types of calcium-dependent protein kinases are present in many animal tissues. These are the calcium/calmodulin-dependent and the calcium/phospholipid-dependent enzymes. It is the latter class that will be discussed here.

The calcium/phospholipid-dependent protein kinase (pk-C) was first reported by Takai and co-workers in 1979, however it was initially discovered as an enzyme that was activated after limited proteolysis by an endogenous calcium-dependent protease (Takai et al. 1977; Inoue et al. 1977). It was recognized that the high levels of calcium required, (well beyond physiological concentrations) and the irreversible nature of the activation, made this unlikely to be a physiologically significant mechanism of regulation. These workers found that the enzyme could also be activated at much lower calcium concentrations by the addition of a membrane fraction. Testing of the effects of various phospholipids on the kinase showed that phosphatidylserine was the most effective activator. Biochemical characterization of the proteolyzed enzyme showed that with total calf thymus histone or histone 2B as substrate, pk-C was active at 75mM $MgCl_2$ and

virtually inactive at 5mM $MgCl_2$. This result was in direct contrast to the cAMP-dependent enzyme (pk-A) which was inactive at 75mM $MgCl_2$ and highly active at 5mM $MgCl_2$. Thus relative activity at 5 and 75mM Mg ,in the presence and absence of cAMP, could be used to distinguish radioactive incorporation due to pk-C from that due to pk-A (Takai et al. 1977;Inoue et al. 1977) In contrast to the proteolyzed enzyme, native pk-C was always more active with 5 mM $MgCl_2$

than with 75mM (Takai et al. 1977;Inoue et al. 1977;Takai et al. 1979a,b).

The chromatographic behavior of pk-C on anion exchange has been described. The mammalian enzyme consistantly elutes from DE52 anion exchange at about 175mM NaCl (Inoue et al. 1977;Takai et al. 1977;Ferris, unpublished results).

Protein kinase C is thought to be involved in regulating growth and differentiation. It has been implicated as the mediator of the tumorigenic effects of phorbolester tumor promoters (Nishizuka 1984). In fact it has been shown that these tumor promoters can directly activate pk-C in much the same manner as phospholipids do (Nishizuka 1984). Others have reported that a rapid translocation of pk-C from the cytosol to the plasma membrane occurs in response to the binding of IL2, or IL3, by T lymphocytes. This translocation event is correlated with mitogenesis and differentiation induction by IL2 (Farrar & Anderson 1985; Farrar et al. 1985) and analogous results have been seen in other systems (Nishizuka 1984; Hirota et al. 1985). It has also been reported that pk-C phosphorylates epidermal growth factor receptor *in vitro* and that factors that activate pk-C *in vivo* also cause phosphorylation of the receptor on the same residues phosphorylated by pk-C *in vitro*. The converse reaction has also been described, that is, activation of pk-C, due to phosphatidylinositol breakdown, promoted by binding of epidermal growth factor to the receptor (Bertics et al. 1985)

While these findings have indicated a role for pk-C during development in some systems, it is uncertain how general a phenomenon it represents. Because of the involvement of cyclic phosphorylation/dephosphorylation events on the development of Dictyostelium discoideum, it is of interest whether pk-C is also present in this organism. We report here on a histone kinase

activity from undifferentiated Dictyostelium that displayed some of the characteristics of mammalian pk-C.

4.2 Materials and Methods

4.2.1 Materials

[γ^{32}P]ATP (25 Ci/mmol) was purchased from ICN. Kemptide was from Calbiochem-Boehring. P 81 filter paper and DE52 cellulose were from Whatman. Sephacryl S300 was from Pharmacia. Rabbit brains were obtained from the Animal Science Dept. All other reagents were from Sigma.

4.2.2 Harvesting of cells and preparation of extracts.

AX3 cells were grown in HL5 medium in two liter flasks on a rotary shaker (Cocucci & Sussman 1970). The cells were harvested by centrifugation in a continuous flow rotor, washed three times and resuspended in 2 volumes (w/v) of 20 mM Tris HCl pH 8.0 containing 2 mM EDTA, 0.5 mM EGTA and 2 mM PMSF (buffer A). A volume of 30% Triton X100 was added to give a final concentration of 0.2%. The mixture was incubated on ice with gentle stirring for 15-20 min. Cell lysis was monitored by phase contrast microscopy and was usually complete in 15 min. The lysate was then centrifuged at 100,000 g for 1hr. In most preparations 8-10 grams of wet cells were used as starting material.

Rabbit brains were obtained fresh from animals killed at the Animal Science facility at VPI &SU. The brains were transported on ice, cut into 10 gram sections and stored frozen at -80 °C. For use, a section was thawed in 20 ml of buffer A and homogenized thoroughly using a

Potter-Elvehjem tissue grinder. 30% Triton X100 was added to give a final concentration of 0.2% and the mixture was homogenized one additional stroke. The homogenate was then incubated 15-20 min on ice as above, then centrifuged at 100,000 g for 1hr.

4.2.3 DE52 chromatography

The soluble fraction from either source was applied to 0.2 volumes of packed DE52 resin that had been equilibrated in buffer A. After stirring on ice for 10-15 min the resin was settled by low speed centrifugation and the supernatant (DE52 flow-through) decanted. The packed resin was loaded onto a column (1.2 x 20 cm) and washed with buffer A until the absorbance at 280nm (as determined by a column monitor) reached baseline. The column was then eluted with a one hour linear 0-200mM NaCl gradient in buffer A.

4.2.4 Protein kinase assays.

Protein kinase C was assayed by mixing 10µl of enzyme fraction with 40µl of a reaction mixture containing 50 mM Tris HCl pH 8.0, with 1mg/ml histone, 10 mM $MgCl_2$, 0.75 mM $CaCl_2$, and 8.6 uM [γ^{32}P]ATP (25 Ci/mmole) plus or minus 96 µg/ml phosphatidylserine and 10µl/ml diolein. After a 3 min incubation at 30 degrees, the reaction mixtures were transferred to 1cm square P 81 filters and passed through two cold 30% and three room temperature 15% acetic acid washes (five min each). After washing the filters were briefly placed in acetone followed by air drying. Radioactivity incorporated was determined by liquid scintillation counting.

4.3 Results and Discussion.

4.3.1 Ca^{2+} and Mg^{2+} effects on kinase activity

Table 4 on page 72 shows the results of an experiment in which Dictyostelium extracts were preincubated either with or without added Ca^{2+}, then assayed for kinase activity with 5 or 75mM $MgCl_2$ The increased incorporation in the presence of 75 mM $MgCl_2$ was repeatable but it did not seem to depend on preincubation with calcium. Since cGMP-dependent protein kinase is activated by high concentrations of $MgCl_2$ (Takai et al. 1977) it seemed possible that the activation might be due to it, however when tested, no cGMP-dependent activity could be demonstrated in any of the preparations. It may be that sufficient endogenous calcium was present to activate the Dictyostelium protease although increasing EGTA two fold in the minus calcium preincubation mixture did not alter the results.

4.3.2 DE52 chromatography.

The elution of the rabbit brain pk-C from DE52 (Figure 14 on page 70) was very reproducible in repeated experiments. However the degree of phospholipid dependency did vary significantly. In three out of ten experiments no dependency on phospholipids could be shown. This may have been due to a time dependent degradation of the enzyme, however, since a fraction that was 6 fold dependent when assayed fresh, was only two fold dependent a day later and completely independent two days later. In many of the experiments, columns were run at night and not assayed until the following morning. The results of one experiment with a Dictyostelium extract are shown in (Figure 15 on page 71).

As with the rabbit enzyme, the amount of salt needed to elute the enzyme was very consistent and virtually identical to the rabbit pk-C. Clear dependency on phospholipids was only seen in the one experiment shown here out of a total of nine separate experiments, although in a few other cases some very slight (much less than two fold) dependency was seen.

These limited data are suggestive, but not convincing, that pk-C is present in Dictyostelium. If this investigation were to be pursued, a good first step would be to determine the molecular weight of the histone kinase, since elution of the mammalian pK-C from gel filtration gives size estimates of 70-100 KDa (closer to 50 kDa for the proteolyzed enzyme). Additional approaches might include using higher levels of EGTA and some specific calcium-dependent protease inhibitors (antipain, leupeptin) in the extraction buffer. The extraction buffer might also be tested with 20-30 mM mercaptoethanol (M.E.) as some laboratories

have routinely included high concentrations of reducing agent in their buffers. Lastly, it would be prudent to assay developing cells as well as amoebae before a negative conclusion is even considered.

The reason that these experiments were not continued was that during the investigation of the histone kinase activity from *Dictyostelium* we discovered a protein kinase activity that appeared in the DE52 flow-through. At first this kinase was studied because it seemed possible that it might be pk-C that did not bind to the DE52 resin. After a few experiments however it was found that the enzyme was phosphorylating endogenous proteins rather than the histone present in the reaction mixture. This finding was quite intriguing because the level of endogenous incorporation was far higher than we had previously seen in unconcentrated column fractions and it suggested the presence in the same fraction of both a kinase and its specific substrate. Since one of the objectives of the laboratory has been to examine the phosphorylated substrate proteins as well as the kinases and phosphatases, the opportunity presented seemed too good to ignore. We therefore decided to pursue the endogenous kinase rather than continue the pk-C investigation.

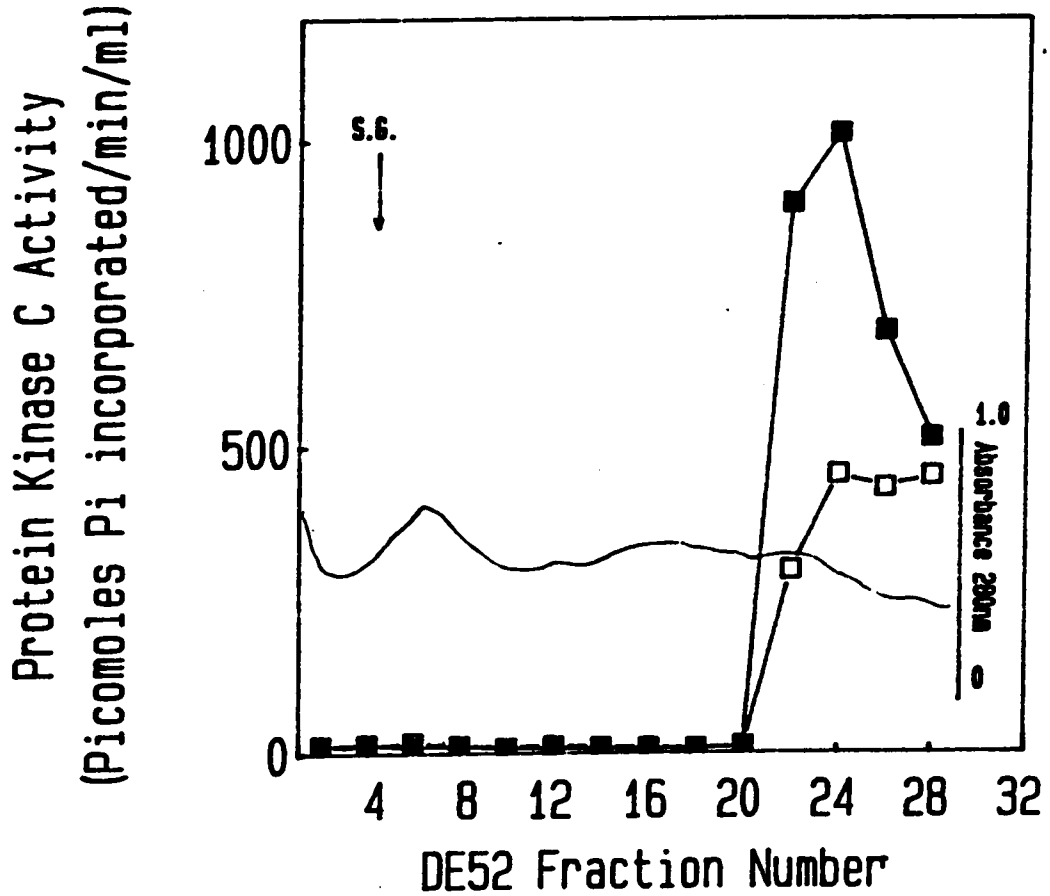

Figure 14. **Elution of rabbit brain pk-C from DE52 resin.:** The column (1.2 x 20cm, flow rate 60ml/hr) was eluted with a linear 1hr 0-200mM NaCl gradient in buffer A. Two ml fractions were collected. Activity, picomoles Pi incorporated min/ml, in the presence (■) and absence (□) of added phospholipids. (—) absorbance at 280nm. S.G, start gradient.

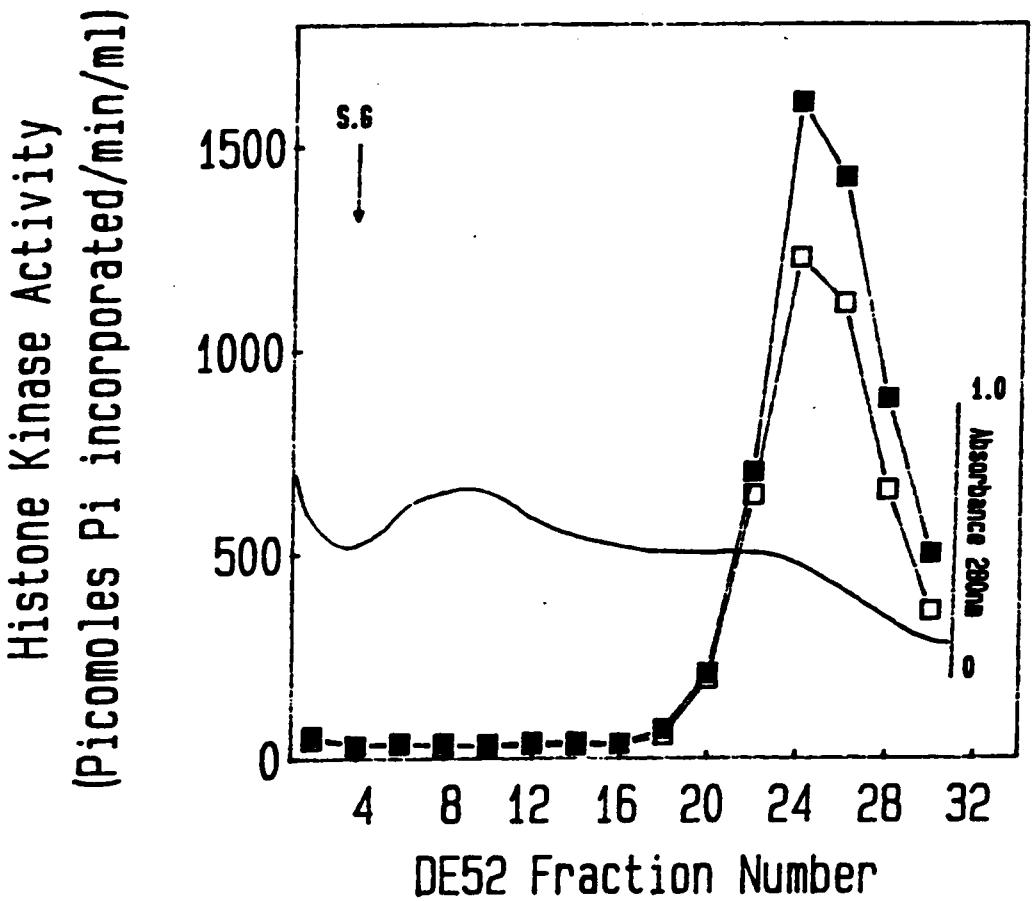

Figure 15. Elution of Dictyostelium histone kinase from DE52.: The column (1.2 x 20cm, flow rate 60ml/hr) was eluted with a 1hr linear 0-200 mM NaCl gradient in buffer A. Two ml fractions were collected. Activity, picomoles Pi incorporated min/ml, in the presence (■) and absence (□) of added phospholipids. (—) absorbance at 280nm. S.G, start gradient.

Table 4. Kinase activity activity with 5 and 75mM $MgCl_2$

	Histone kinase activity in the presence of	
Preincubation conditions	5mM Mg	75mM Mg
-Ca	7103	12330
+Ca	6775	12870

5. Purification and characterization of an autophosphorylating kinase

5.1 Introduction

In the 18 years since the discovery of the cAMP-dependent protein kinase by Krebs and co-workers (Walsh et al. 1968) it has become generally accepted that cyclic phosphorylation of proteins is the single most important mechanism for regulating cellular metabolism. Protein kinases and phosphatases have been found in every phyla of the plant and animal kingdoms that have been investigated and it would be difficult to even mention a major cellular function in which regulation by cyclic phosphorylation does not occur.

In this lab we have been investigating the involvement of cyclic phosphorylation events in regulating the cellular differentiation of *Dictyostelium discoideum*. The importance of cAMP to this process has been known for many years (Konijn et al. 1968). Cyclic AMP functions as the chemotactic agent during aggregation but it is important during later development as well. Its involvement has been shown in a variety of ways. Previous workers have demonstrated that some developmentally regulated enzymes are induced precociously in the presence of exogenous cAMP

(Town & Gross 1978). Lodish and co-workers (Mangiarotti et al. 1982) have shown that addition of cAMP to disaggregated cells specifically stabilizes late stage mRNAs that are otherwise rapidly degraded. In addition, cAMP was found to reinitiate the synthesis of mRNAs which had been degraded in the absence of cAMP. Others have shown that the addition of cAMP to cells that have been prevented from forming cell contacts can be sufficient for differentiation to occur in the absence of normal morphogenesis (Chia 1975).

In an attempt to understand the mechanism by which cAMP effects are mediated to cellular events that occur during development, this laboratory has reported on a cAMP-dependent protein kinase from *Dictyostelium* (Rutherford et al. 1982; Rutherford et al.1984) and on endogenous proteins that were phosphorylated in vitro in a cAMP-dependent manner (Frame and Rutherford 1984). More recently we described the chromatographic separation of phosphatases that dephosphorylated proteins that had been phosphorylated by the cAMP-dependent protein kinase *in vitro* (Ferris and Rutherford 1986). While cAMP and the cAMP-dependent protein kinase are obviously important in the development of *Dictyostelium* it seems unlikely that only cAMP mediated phosphorylation is involved. In mammalian cells multiple phosphorylation systems are associated with the regulation of growth and differentiation including a cGMP dependent (Walter 1981), a Ca/phospholipid dependent (Farrar et al. 1985) and other kinases (Nakamura et al. 1983).

One particularly important event during the differentiation of *Dictyostelium* that may be regulated by phosphorylation is the conversion of glycogen to the end products of development (Marshall et al. 1970; Killick & Wright 1974). In this organism, glycogen metabolism is linked more closely with the process of differentiation rather than with energy production. Given the association of glycogen metabolism with terminal differentiation of the two cell types, the regulation of glycogen synthesis and degradation is of interest. In mammals two forms of glycogen phosphorylase are present that can be interconverted by phosphorylation or dephosphorylation. The phosphorylated "a" form degrades glycogen while the dephosphorylated "b" form is inactive unless its allosteric activator, 5'AMP, is present. Until quite recently only one form of the enzyme, which was 5'AMP-independent, was thought to exist in *Dictyostelium*. A second form of glycogen phosphorylase, that is 5'AMP-dependent, has now been reported (Rutherford 1986) but it is not

certain that these two forms result from phosphorylation and dephosphorylation of a single protein species. The two forms in *Dictyostelium* are developmentally regulated with the 5'AMP-dependent form being present early and the 5'AMP-independent form gradually appearing in later stages (Rutherford and Cloutier 1986).

During experiments directed in part toward demonstrating the presence of phosphorylase kinase in *Dictyostelium* several protein kinase activities were chromatographically resolved. I found that one kinase activity, present in sephacryl 300 fractions was phosphorylating endogenous proteins almost exclusively rather than the substrate protein added to the reaction mixtures. Although phosphorylation of endogenous proteins in *Dictyostelium* had been studied previously by members of this laboratory (Frame & Rutherford 1984) , both the speed of this reaction and the level of incorporation were remarkably high. The amount of radioactivity incorporated was 5-10 fold greater than I had seen in the past in *Dictyostelium* extracts and the reaction usually reached completion in less a minute. This implied that a protein kinase and its specific substrate were both present in those fractions. Because one of my goals has been to study phosphorylated substrate proteins as well as the protein kinases and phosphatases, I decided to further characterize the activity. Further characterization indicated that a prominent low molecular weight protein in the kinase fractions was also the major phosphorylated species. Repeated attempts to either selectively inactivate the kinase, while maintaining the substrate in a form that could be phosphorylated, or to physically separate the substrate from the kinase by ultrafiltration and column chromatography, were unsuccessful. Therefore, I concluded that the low molecular weight phosphorylated protein was itself the protein kinase. Here I report on the purification to homogeneity, and characterization of a previously undescribed protein kinase from *Dictyostelium*. This putative autophosphorylating enzyme, is the most prominent protein in relatively crude sephacryl 300 fractions and it therefore represents a major cellular protein. While its physiological function is uncertain, its mere abundance requires that the role of this kinase during development be understood.

5.2 *Materials and Methods*

5.2.1 Materials

[γ ^{32}P]ATP (25 Ci/mmol) was purchased from ICN. Kemptide was from Calbiochem-Boehring. P 81 filter paper and DE52 cellulose were from Whatman, Sephacryl S300 was from Pharmacia, and Sephadex G100 and all other reagents were from Sigma.

5.2.2 Harvesting of cells and preparation of extracts.

Dictyostelium discoideum (AX3) was grown in liquid HL5 media (Cocucci & Sussman 1970) on a rotary shaker as previously described (Ferris & Rutherford 1986). When differentiated cells were required the amoebae were washed free of media, diluted 1:3 (weight:volume) in MES buffer and plated on non nutrient agar (Rutherford 1976). The cells were harvested as previously described (Rutherford 1976; Ferris and Rutherford 1986) except that buffer A was 20mM Tris HCl pH 8.0 containing 2mM EDTA, 0.5mM EGTA and 50µM PMSF. All subsequent steps were carried out at 4°C. After harvesting the cells were resuspended in 5 volumes of cold buffer A. Undifferentiated cells were immediately sonicated, while differentiated cells were homogenized three strokes with a Potter-Elvehjem tissue grinder prior to sonication. The cells were broken by three 45-s exposures to a 2 cm probe of a sonic cell disrupter (Model 300, Fisher) at a setting of 45 and then centrifuged for 60min at 100,000g.

5.2.3 Column chromatography

The 100,000g supernatant was batch treated with 0.1 volume of DE52 resin that had been equilibrated in buffer A. After stirring 10 min the resin was settled by centrifugation. The supernatant (DE52 flow through) was concentrated with solid ammonium sulfate (70% saturation), the pellet dissolved in 5-6ml of buffer A, and dialyzed overnight against 1 liter of buffer A. The dialysate was clarified by a 10 min 10,000g centrifugation, then was loaded onto a sephacryl 300 column (1.6 x 84cm, flow rate 30ml/hr). The column was eluted with buffer A containing 50 mM KCl; 2 ml fractions were collected. Active fractions from the S300 were pooled, diluted 1:3 with 20 mM potassium phosphate buffer pH 7.0 (Buffer B) and batch treated with 0.1 volume of hydroxylapatite (HA) resin that had been equilibrated in buffer B. After stirring gently 5 min the resin was loaded onto a column (1.2 x 20cm, flow rate 60ml/hr) and washed with buffer B. When the absorbance at 280 nm returned to baseline, as determined by a column monitor, the column was eluted with a 2 hr linear 20-600 mM potassium phosphate (pH 7.0) gradient . Two ml fractions were collected. Active fractions from the first HA column were pooled, diluted 1:5 in buffer B and batch treated with 0.1 volume of HA as described above. The second HA column was loaded with the resin and eluted as described above, except that the gradient was 20-300 mM potassium phosphate.

5.2.4 Preparative SDS polyacrylamide gel electrophoresis.

The active fractions from the second HA column were mixed with 100ul/ml fraction of a concentrated SDS stop solution (20% SDS, 50% dithiothreitol, 0.02% pyronin) and 25-30 sucrose crystals. The mixture was boiled for 3 min, then the entire volume was loaded onto a 1.6mm thick 15 % preparative slab gel with a 1 cm 6% stacking gel. Electrophoresis was at 20mA until the tracking dye moved into the resolving gel, then continued at 40mA. The electrophoresis buffer (pH

8.75) was 50mM Tris, 380mM glycine and 0.1% SDS. After electrophoresis the gel was stained with Coomassie for 8 min, then destained for 10 min with gentle heating on a hot plate to speed the process. The strip containing the protein was cut from the gel and eluted overnight with electrophoresis buffer using an Isco electrophoretic sample concentrator (model 1750) set at 3mA. After elution from the gel, the protein was diluted in 10 ml of 40 mM Tris HCl pH 8.6 and reconcentrated in the same buffer using the electrophoretic concentrator.

5.2.5 Protein Kinase Assays.

Phosphorylation of endogenous proteins was tested by mixing 25µl of column fractions with 25 µl of a reaction mixture consisting of 50 mM Tris HCl pH 8.0, 1mM $MgCl_2$and 1.6 µM [γ^{32}P]ATP (25 Ci mmole). After incubation for 3 min at 23 ° C the mixtures were transferred to 1 cm square P 81 filters. The filters were washed twice in 300 ml cold 30% acetic acid, three times in 300 ml room temp. 15% acetic acid and once in 300 ml acetone (5 min each). After air drying, the amount of radioactivity incorporated was determined by scintillation counting.

Casein kinase was assayed as above except the reaction mixture consisted of 50 mM Tris HCL pH 7.5, 5mM MgCl, 5mM $MnCl_2$, 5mg/ml casein, and50µM [γ^{32}P]ATP (5Ci mmole).

5.2.6 Photoaffinity labeling with [α^{32}P]ATP

The enzyme was photoaffinity labeled by mixing 12 µl of enzyme with 3 µl of 0.4µM [α^{32}P]ATP (25 Ci/mM) on a layer of parafilm placed over ice. The reaction mixture was illuminated by a Blak-Ray high intensity ultra violet lamp (UVS 54) from a distance of 10 cm for 30 sec. Following illumination the reaction was stopped by the addition of 5µl of the concentrated SDS stop solution and 30 µl of double distilled water (ddH_2O). The samples were then subjected to SDS polyacrylamide gel electrophoresis.

5.2.7 Analytical SDS PAGE, Autoradiography and Immunoblotting

SDS PAGE and autoradiography was performed according to the method of Rudolph & Krueger (1979). Samples (40ul) were applied to the slab gels made with either 7.5, 10 or 15 % resolving gels and 6% stacking gels (0.8mm thick). Proteins were silver stained or transferred to nitrocellulose sheets. Transfer was accomplished using a Hoefer Scientific Instruments power source (model TE51) set at 1.2A for one hour. The transfer buffer was 25mM Tris, 192mM glycine and 20% methanol, pH 8.3. After transfer, the nitrocellulose was placed in 50 ml of 50mM Tris, 200mM NaCl, 0.05% Tween 20 (TBS-tween) and gently shaken for 20-30 min. The buffer was then replaced with 50 ml of fresh TBS-tween and 300ul of the antiserum was added. After an overnight incubation with the antibody, the nitrocellulose was rinsed several times with fresh 50ml solutions of TBS-tween. A 50 ml solution of TBS-tween containing 1ug/ml protein A peroxidase was then placed onto the blot and incubated with gentle shaking for 1hr. The blot was rinsed with two 50ml volumes of TBS-tween and then with 50 ml of TBS. The blot was then exposed to 48mls of peroxidase reaction mix containing 8ml of 3mg/ml 4 chloro-1-napthol, 40 ml TBS and 14ul 30% H_2O_2 for 30-120 min. In some cases the blots were also stained for protein using 0.1% napthol-blue black (amido black) in 45% methanol and 10% acetic acid. 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.

5.2.8 Antibody Preparation

A New Zealand white rabbit was injected with 150µg of the purified protein in complete Freunds adjuvant. The rabbit was 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 10,000g for 10 min. The serum was divided into 300ul aliquots and stored frozen at -20 °C for subsequent use.

Preparation of casein kinase

Casein kinase was prepared by harvesting and breaking the cells as described above but the buffer was 50mM Tris HCl pH7.5 containing 0.02% sodium azide, 2mM mercaptoethanol, and 2mM benzamidine (buffer C). The soluble extract was batch treated with DE52 as described for the endogenous protein kinase, except using buffer C. The resin was loaded into a column (4.5 x 4.0cm, flow rate 160ml/hr) and eluted with a linear 8hr 0-0.3M KCl gradient in buffer C.

5.3 Results

5.3.1 Purification of an autophosphorylating protein kinase

Stationary phase amoebae (10-20g wet wt) were harvested and prepared for column chromatography as described in Methods. When the 100,000g supernatant was applied to DE52 cellulose chromatography the majority of the endogenously phosphorylated protein was found in the flow-through volume. This volume was concentrated and applied to a S300 column. The concentrated DE52 flow-through was resolved into two peaks of endogenous phosphorylation by the S300 column (Figure 16 on page 82). The first peak of endogenous activity eluted just following the void volume indicating a molecular weight in excess of 300 KDa. This activity was labile and in some cases difficult to resolve from the second peak which was quite stable. The elution volume of the second peak indicated a molecular weight of 60-100 KDa. When the column

was assayed for casein kinase little incorporation that depended on the presence of casein was found. We also tested various types of histones as well as Kemptide and protamine sulfate as potential substrates for this kinase activity. None of these potential substrates increased the incorporation significantly over the endogenous incorporation when assayed in the endogenous kinase reaction mixture.

When the S300 fractions containing the second peak of activity were applied to hydroxyapatite (HA) resin, virtually all of the activity was retained. A potassium phosphate gradient to 600mM, eluted a single sharp peak of activity that corresponded to a peak of absorbance at 280 nm (Figure 17 on page 83). The fractions containing activity from the first HA column were loaded onto a second HA column. No activity appeared in the flow-through. A single sharp peak of absorbance at 280 nm was eluted in the gradient, that corresponded with the endogenous phosphorylation activity (Figure 18 on page 84). Samples of the fractions across the peak of activity were incubated for two min in endogenous phosphorylation mixture and then subjected to SDS PAGE and autoradiography. Only a single dark band was observed on the film, and that corresponded to a prominent low molecular weight protein on the silver stained gel (Figure 19 on page 85)

One ml of the peak fraction from the second HA column was loaded onto a preparative SDS gel. Following electrophoresis and staining, the strip containing the low molecular weight protein was cut from the gel and eluted. When the eluted material was subjected to SDS PAGE and silver staining a single protein band of about 20 KDa (pp20) was seen (Figure 20 on page 88). The final preparation contained 250ug of pure protein that represented a minimum of 0.03% of the total cellular protein This purified material was used as an antigen for preparation of antibodies. The specific activity of the purified enzyme could not be determined since after SDS PAGE it had no activity.

A sample of the second HA activity peak was applied to nondenaturing tube gels. After electrophoresis, three major bands were observed. A duplicate gel was sliced into sections corresponding to the three protein bands, placed into three tubes and covered with 1ml of buffer B. After three hours the tubes were assayed for endogenous phosphorylation. The tube with the

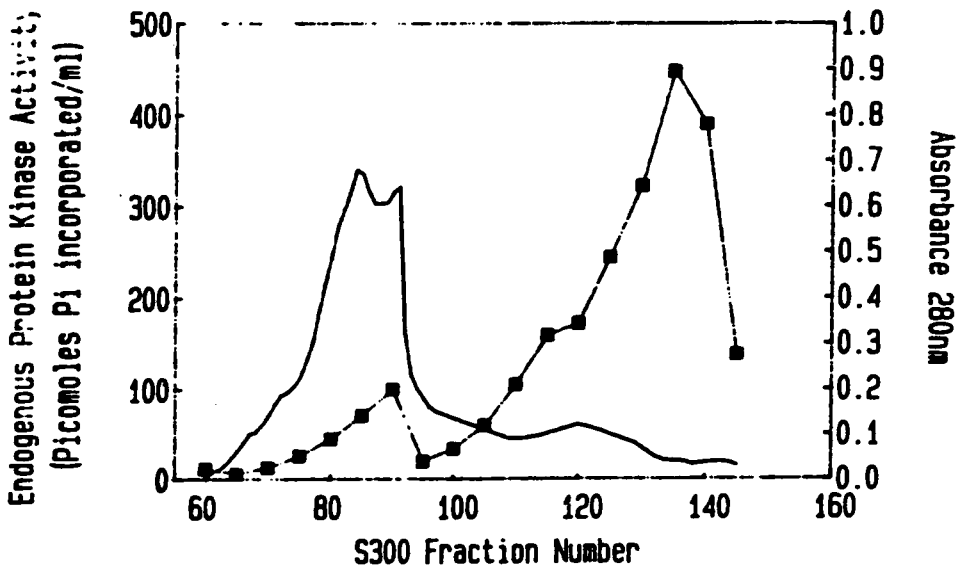

Figure 16. S300 Endogenous protein kinase: The column (1.6 x 84cm, flow rate 30ml/hr) was loaded with 2ml of the concentrated DE52 flow-through. One ml fractions were collected. (■) Endogenous protein kinase activity. (—) Absorbance at 280nm.

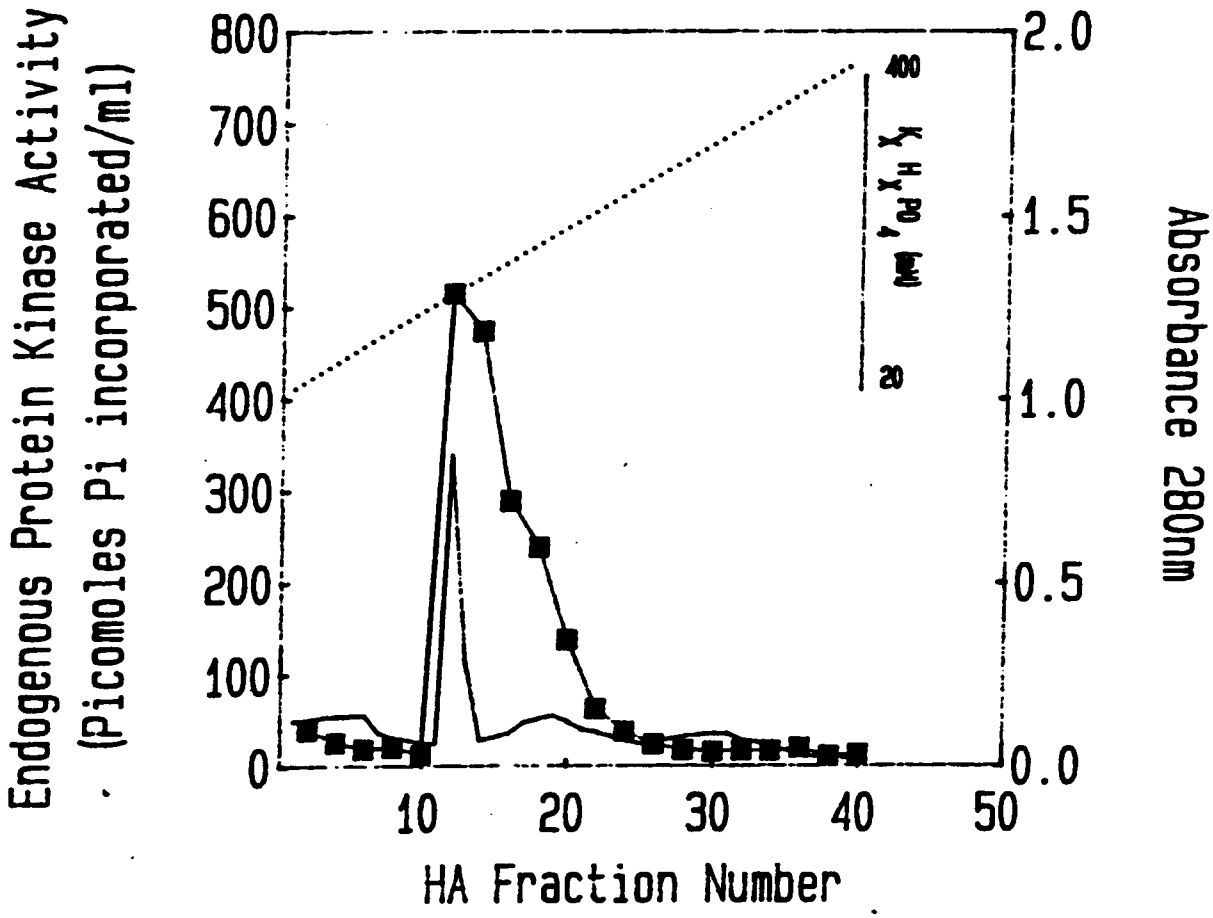

Figure 17. **First hydroxylapatite, endogenous protein kinase:** The column (1.2 x 20cm, flow rate 60ml/hr) was eluted with a 2hr linear 20-600mM potassium phosphate gradient. Two ml fractions were collected. (▪) Endogenous kinase. (—) Absorbance at 280nm.

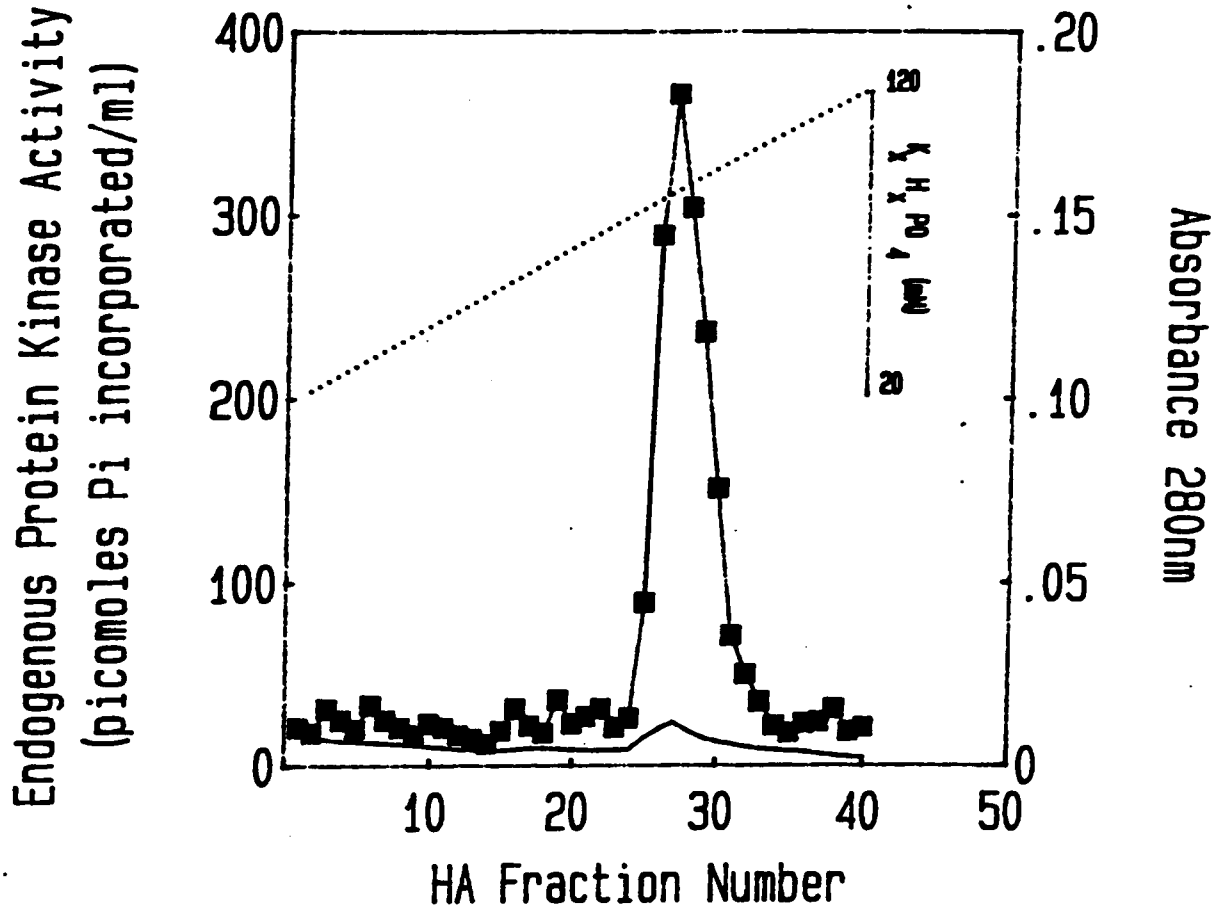

Figure 18. **Second Hydroxylapatite, endogenous protein kinase:** The column (1.2 x 20cm, flow rate 60ml/hr) was eluted with a linear 2hr 20-300mM potassium phosphate gradient. Two ml fractions were collected. (▪) Endogenous kinase. (—) Absorbance at 280nm.

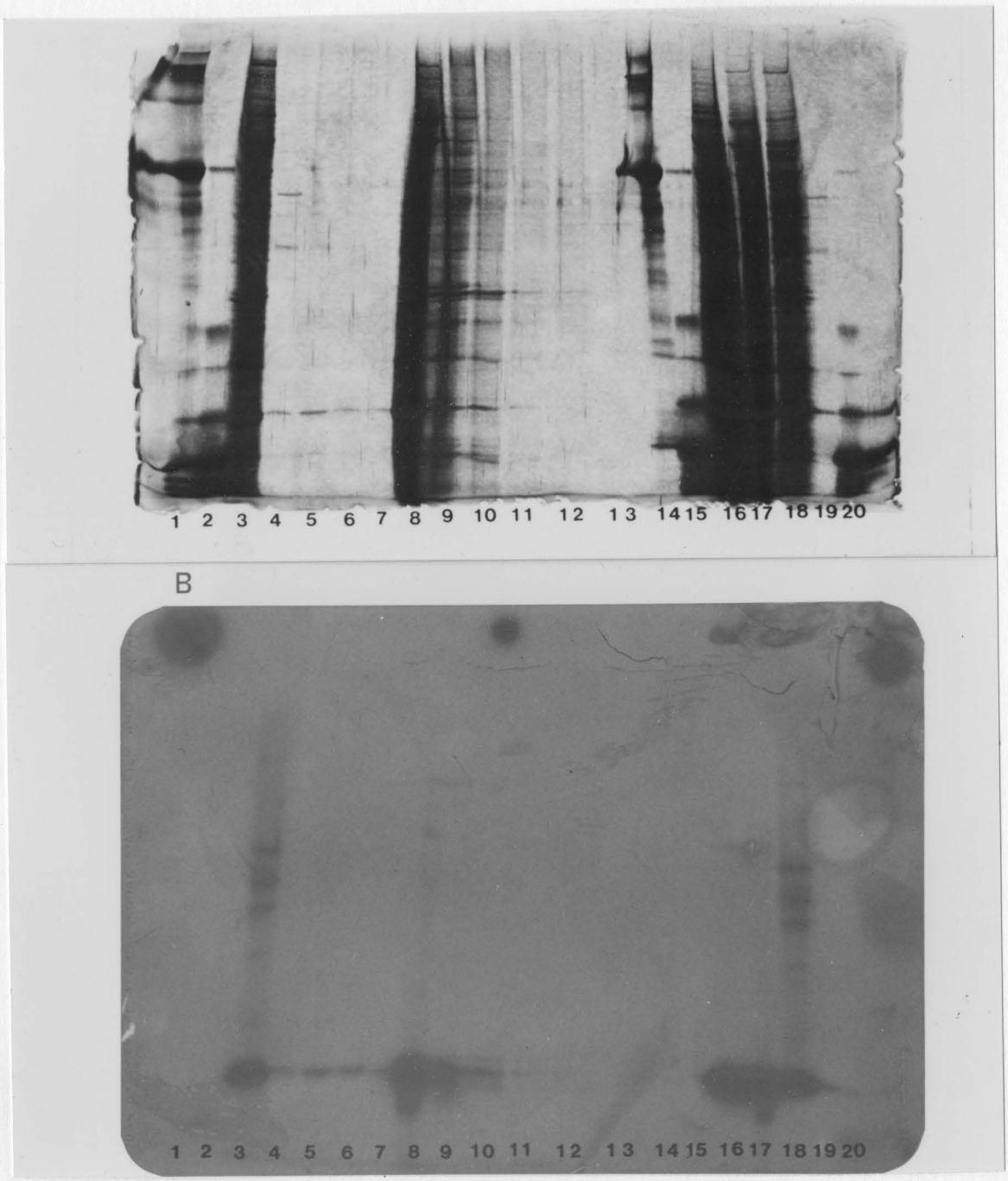

Figure 19. **Gel and autoradiograph across HA1 peak:** (A) Silver stained 12% gel (B) Autoradiography Lanes 1-4 are BSA (67KDa), Lactoalbumin (14.2 KDa), "On" column, and flow-through, respectively. Lanes 5-13 are fractions 6, 8, 10, 12, 14, 16, 18, 20, and 22 respectively. Lanes 14 and 15 are BSA and Lactoalbumin respectively. Lanes 16-17, fraction 12. Lanes 18-20 are "on", Flow-through, and Lactoalbumin, respectively.

slowest migrating band contained the only significant activity. Because it was possible that separation of kinase and substrate had occurred during electrophoresis, samples of the eluted gel slices were mixed together and assayed for endogenous phosphorylation. No additional incorporation was seen in the mixed samples. This result was in agreement with results from earlier experiments involving the mixture of various column fractions together to locate substrates that had become separated from the kinase during chromatography. If a fraction containing a large amount of substrate but little or no kinase was mixed with one containing kinase but little substrate, incorporation in the mixture would be much greater than the additive amount of incorporation in the separated fractions. But no incorporation greater than additive values resulted from any of these experiments. This indicated that either the substrate exhibited chromatographic and nondenaturing electrophoretic behaviors very similar to the kinase (or perhaps that the substrate was tightly bound to the kinase), or that the incorporation was due to autophosphorylation. The proteins that were eluted from the tube gel slices were then exposed to reaction mixture and then subjected to SDS PAGE and autoradiography. The slowest migrating band from the tube gel gave rise to one major protein (pp20) on the silver stained SDS gel and that corresponded to the only band visible on the autoradiograph (lane five, Figure 21 on page 89). To further test whether the phosphorylation was due to autophosphorylation I tested the radioactive incorporation in a wide range of dilutions of hydroxylapatite fractions (not shown). The rational being that if the incorporation did not result from an intramolecular reaction, then dilution should slow down the rate of the reaction). In these experiments I found no change in the kinetics of the reaction although the total incorporation diluted in a linear fashion. This suggested that pp20 was the subunit of a larger enzyme or aggregate, (estimated M&subr. from gel filtration was 60-100KDa) and that it autophosphorylated by an intramolecular reaction.

If a protein is the catalytic subunit of a protein kinase then it must contain an ATP binding site. To determine if pp20 contained an ATP binding site we carried out a photoaffinity labeling experiment using alpha labeled ATP. Since in the protein kinase reaction only the γ or terminal phosphate of ATP is transferred, the demonstration of radioactivity incorporated in the presence

of α labeled ATP, proves the presence of an ATP binding site. Figure 22 on page 90 shows that pp20 was labeled by alpha ATP and therefore did contain an ATP binding site.

5.3.2 Time course of phosphorylation and dephosphorylation

Autophosphorylation of a protein kinase may represent a regulatory mechanism, but if its activity is to reversibly regulated there must also be a mechanism for dephosphorylation.

Figure 23 on page 92 shows a time course of radioactive incorporation in samples of the DE52 flow-through and in a sephacryl 300 fraction. With the DE52 flow-through preparation the incorporation reached a peak by 2 min and during the next 14 min the amount of incorporation steadily declined. In contrast, when the same experiment was performed after sephacryl 300 chromatography, the incorporation was stable from 2 through 20 min. This suggests that a protein phosphatase is present in the DE52 flow- through, that recognizes phospho pp20 as a substrate, and that the phosphatase is separated from the kinase during gel filtration. Thus the potential exists for reversible phosphorylation of pp20.

5.3.3 Cation requirements for endogenous phosphorylation

Kinase enzymes require one or more cations, often in millimolar concentrations for optimal activity. In most cases Mg^{2+} is the preferred cation although Mn^{2+} often will substitute to some extent. Some kinases, such as the Ca^{2+}/calmodulin-dependent protein kinase, require both Mg^{2+} and an additional cation for activity. To determine which cations best supported phosphorylation of pp20, a peak fraction from the second HA column was tested with Ca^{2+}, Mg^{2+}, and Mn^{2+}. I found that the incorporation that occurred in the absence of exogenous cations could be inhibited by the inclusion of 5mM EDTA (final concentration) in the reaction mixture, while 5mM EGTA had no effect. The inhibition by EDTA could be relieved by adding 10mM Mg^{2+} or Mn^{2+} or

Figure 20. **Purity of final protein preparation:** The 10% gel was silver stained and overdeveloped to detect contaminating proteins. After preparative SDS PAGE the strip containing the protein was electrophoretically eluted and the eluted protein concentrated to 500µl with the electrophoretic concentrator. Lanes 1-2, 20µl of the electrophoretically concentrated protein was applied/lane. Lanes 3-4, a 400µl volume of the concentrated protein was precipitated with 5ml of cold acetone, and resuspended in 200µl of water. 20µl was loaded/lane.

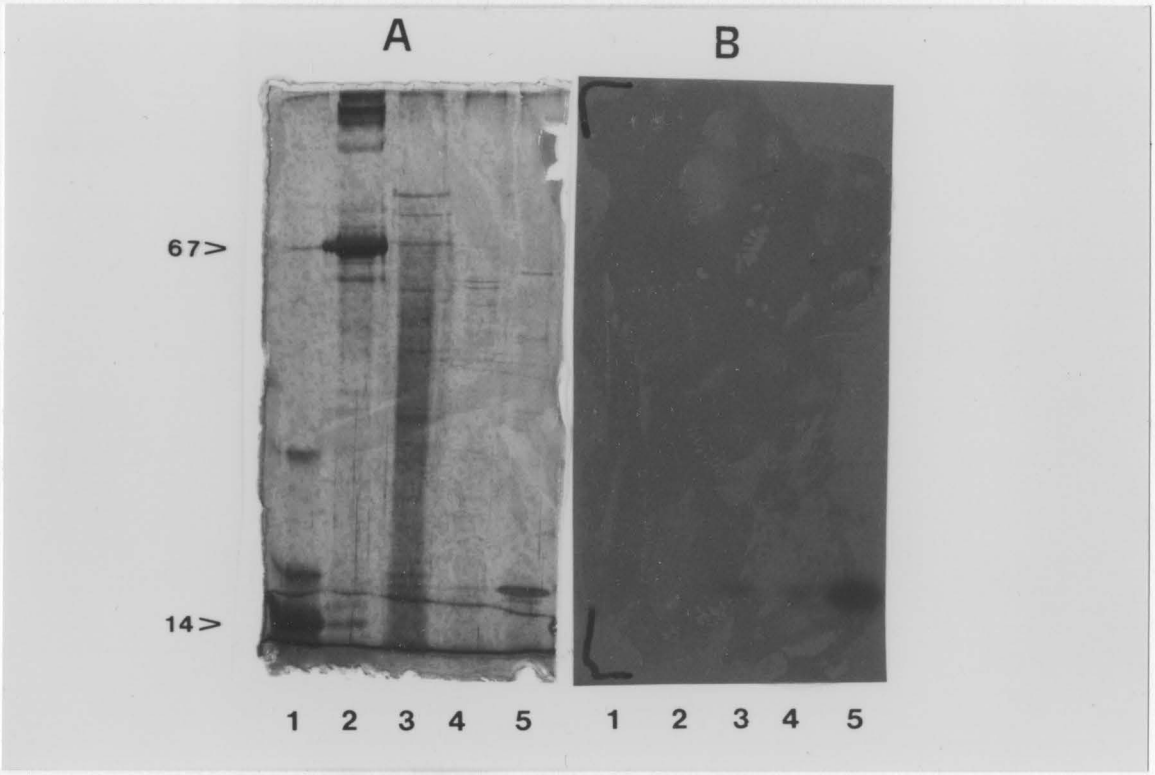

Figure 21. **Protein and kinase activity in nondenaturing tube gel slices.:** (A) Silver stained 10% gel (B) Autoradiography, 1. BSA 2. Lactoalbumin, 3. Fast migrating protein from tube gel. 4. Intermediate migrating protein from tube gel. 5. Slow migrating protein from tube gel. 40µl volumes were applied/lane.

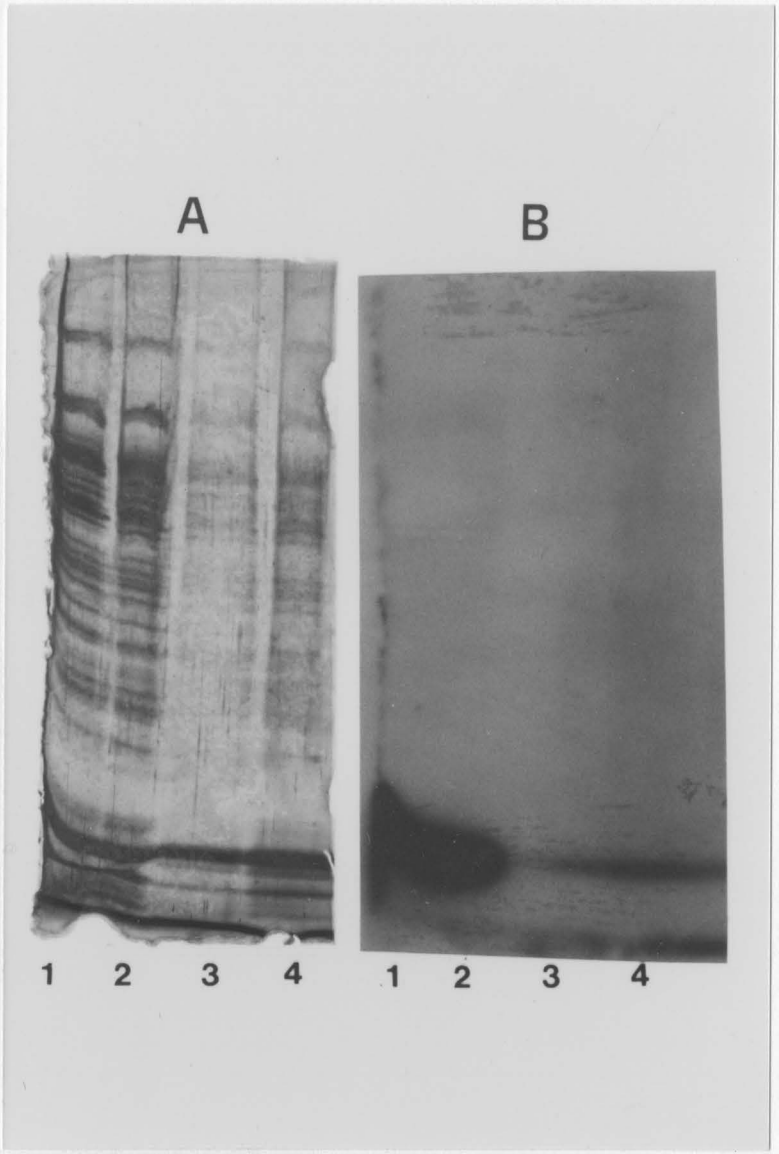

Figure 22. **Demonstration of ATP binding site by photoaffinity labeling:** (A) Silver stained gel. (B) Autoradiography. 1 and 2. Fraction 27 from the second HA column was incubated with [&gama.-^{32}P]ATP under the conditions used for photoaffinity labeling. 3. A 1:10 dilution of fraction 27 incubated with [α-^{32}P]ATP. 4. A 1:5 dilution of fraction 27 incubated with [α-^{32}P] ATP

Ca^{2+}. (Figure 24 on page 93). Similar results were obtained when fractions from the two peaks of activity from the S300 column were tested. I concluded that the kinase activity in the absence of exogenously added cations was due to some tightly bound cation that remained with the protein during purification even though the extraction and S300 column buffer (buffer A) contained 2 mM EDTA and 0.5 mM EGTA. In addition it seemed clear that the bound cation was not calcium since the reaction was not inhibited by 5 mM EGTA but was inhibited by 5 mM EDTA,although calcium did recover the activity after inhibition by EDTA. In other experiments (not shown) I found that in the absence of EDTA in the reaction mixture, the addition of either 5 mM Mg^{2+} or Mn^{2+} actually inhibited the incorporation by up to 80%. That finding may explain why this activity had escaped detection in previous investigations.

5.3.5 Relationship of pp20 to a 85 KDa protein.

The antibody in 6 week post injection serum recognized pp20 while preimmune serum did not. The blot and autoradiograph in -- Figure id " unknown --shows that the antibody recognizes the same 20KDa protein (pp20) that is phosphorylated. When 100,000g supernatants were subjected to western blotting two bands were recognized by the antibody, a lower band, (pp20) corresponding to what had been injected into the rabbit and a much higher band (85 KDa),(Figure 26 on page 97). The figure also shows that both proteins are present throughout the development of *Dictyostelium* and that the amounts of pp20 and the 85 KDa protein (pp85) seem to be consistent from one stage to the next until the fruit stage, when both pp20 and pp85 are substantially reduced. Although in the 100,000g supernatant only pp20 and pp85 were immunostained, multiple bands, intermediate in size between pp20 and pp85, were recognized in S300 column fractions. An association between the relative amounts of pp85 and pp20 in S300 fractions is shown in Figure 27 on page 98In S300 fractions 85 and 90 there is little pp20 while several bands corresponding to the position of pp85 are quite prominent. However, in fractions 105-135, there is a direct correspondence between the amounts of pp20, pp85 and of a protein that migrates

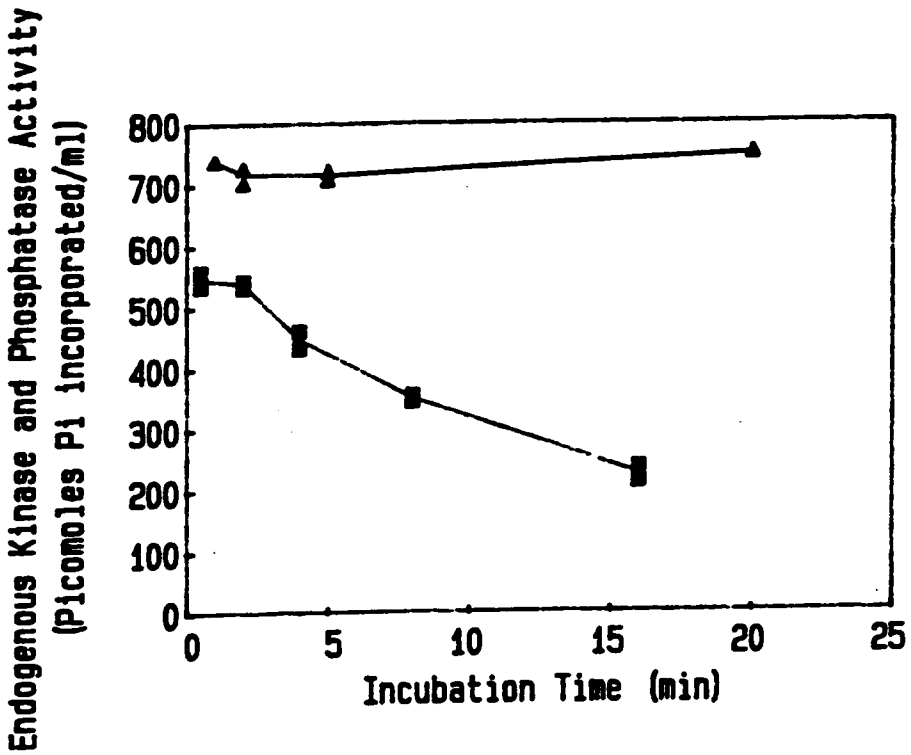

Figure 23. Time courses of phosphorylation and dephosphorylation in the DE52 flow-through and in S300 fraction 135 (from fig 1): 25ul volumes of the samples were mixed with 25ul of endogenous phosphorylation reaction mixture and incubated for the indicated times. (■) Incorporation in DE52 samples. (▲) Incorporation in S300 fraction 135.

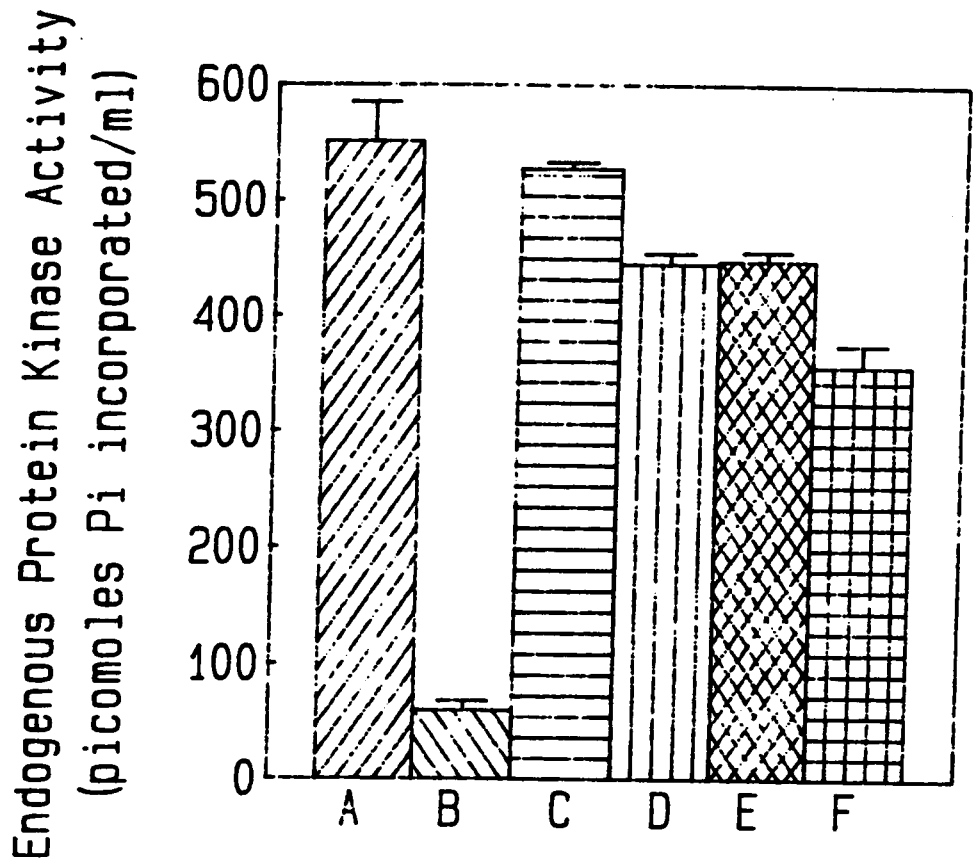

Figure 24. **The effects of cations on endogenous phosphorylation.:** HA #2 fraction 27 (from fig 3) was tested to determine cation requirements. The enzyme samples were incubated 2min. in the presence of endogenous phosphorylation mixtures containing A. No exogenous cation B. 5mM EDTA C. 5mM EGTA D. 5mM EDTA + 10mM Ca^{2+} E. 5mM EDTA + 10mM Mn^{2+} F. 5mM EDTA + 10mM Mg^{2+}

midway between them (pp40). This suggested that these proteins were related in some fashion, possibly, that pp40 and pp20 were proteolyzed fragments of pp85, or that pp40 and pp85 represented dimeric and tetrameric forms respectively, of pp20. The elution volume of the fractions in lanes 11-12 suggests an apparent molecular weight of 60-100 KDa which is consistent with the mobility of pp85 on SDS PAGE. Clearly pp20, which also peaks in those same fractions, must aggregate or be associated with additional protein in order to elute at that volume.

I also tested the cross reactivity of anti pp20 to a a casein kinase from *Dictyostelium*. This casein kinase eluted from DE52 resin at about 150mM NaCl at pH 7.5. When DE52 gradient fractions containing casein kinase activity were subjected to western blotting, a protein of 85 KDa was intensely stained by anti pp20 (Figure 28 on page 99) I have attempted to convert pp85 both from 100,000g supernatants and from DE52 casein kinase preparations into pp20 by proteolysis with beta chymotrypsinogen (Figure 29 on page 100). In panel A, the digestion of a 100,000g supernatant, resulted in the complete loss of pp85, but had little effect on pp20; certainly it did not increase the amount of pp20. In panel B, digestion with increasing amounts of chymotrypsinogen generated a series of bands recognized by the antibody (lane 5), but it did not convert pp85 into a 20 KDa protein (lane 6 contains undigested pp20 immunoprecipitated from an HA fraction). The blot shown in panel B was first immunostained (black bands) then stained for protein (grey bands) with amido black.

5.3.6 Immunological similarity of pp85 and rabbit phosphorylase kinase

My colleagues (Venil Naranan & Debra Brickey, unpublished results) have succeeded in demonstrating phosphorylation of *Dictyostelium* glycogen phosphorylase "b" *in vitro* by incubating "b" enzyme preparations that also contained casein kinase activity, with γ labeled ATP. This suggested that the casein kinase might could also have phosphorylase kinase activity. I tested this possibility by using an antibody prepared against rabbit phosphorylase kinase in conjunction with

anti *Dictyostelium* pp20/85 to investigate the immunological similarity of *Dictyostelium* pp85 to rabbit phosphorylase kinase.

Dictyostelium soluble extracts were immunoprecipitated using anti *Dictyostelium* pp20/85 and with antibody prepared against purified rabbit phosphorylase kinase (Lawrence et al. 1986). When the blot was stained with anti pp20/85, similar 80-90 KDa proteins were recognized in both immunoprecipitates (Figure 30 on page 101). Commercial rabbit phosphorylase kinase was also subjected to SDS page and western blotting (Figure 31 on page 102). A sample of the commercial enzyme was further purified by elution from a sephacryl 300 column (lanes 9-17). The absorbance at 280 nm and the phosphorylase kinase activity both peaked in the same fraction (lane 13) where the greatest amount of protein was immunoprecipitated and immunostained by anti pp20/85. The bands in the commercial preparation stained by anti pp20/85 migrate at a position close to that of pp85 from *Dictyostelium* (lanes 4 & 5). Thus *Dictyostelium* pp85 was close in size to and immunologically similar to a protein present in rabbit phosphorylase kinase preparations.

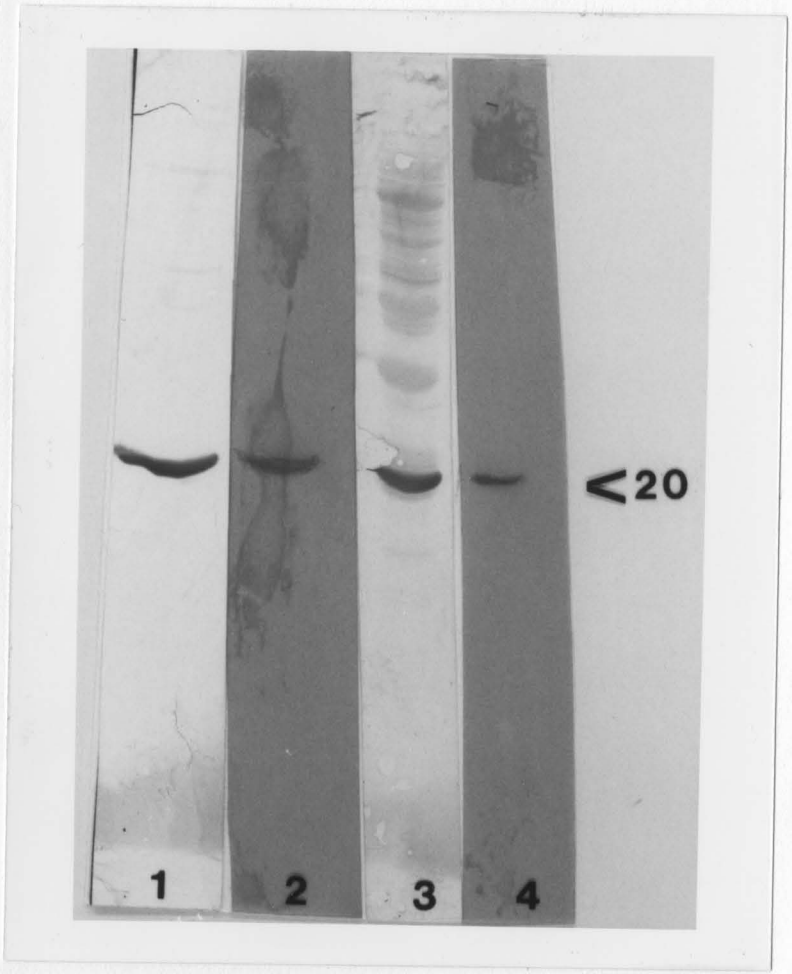

Figure 25. **Recognition of the 20 KDa phosphorylated protein by immune serum:** Enzyme samples (HA F27 from fig 3) were incubated with endogenous phosphorylation reaction mixture followed by SDS PAGE, western blotting, immunostaining and 72 hr autoradiography. Lanes 1 and 3. Western blot Lanes 2 and 4. Autoradiography.

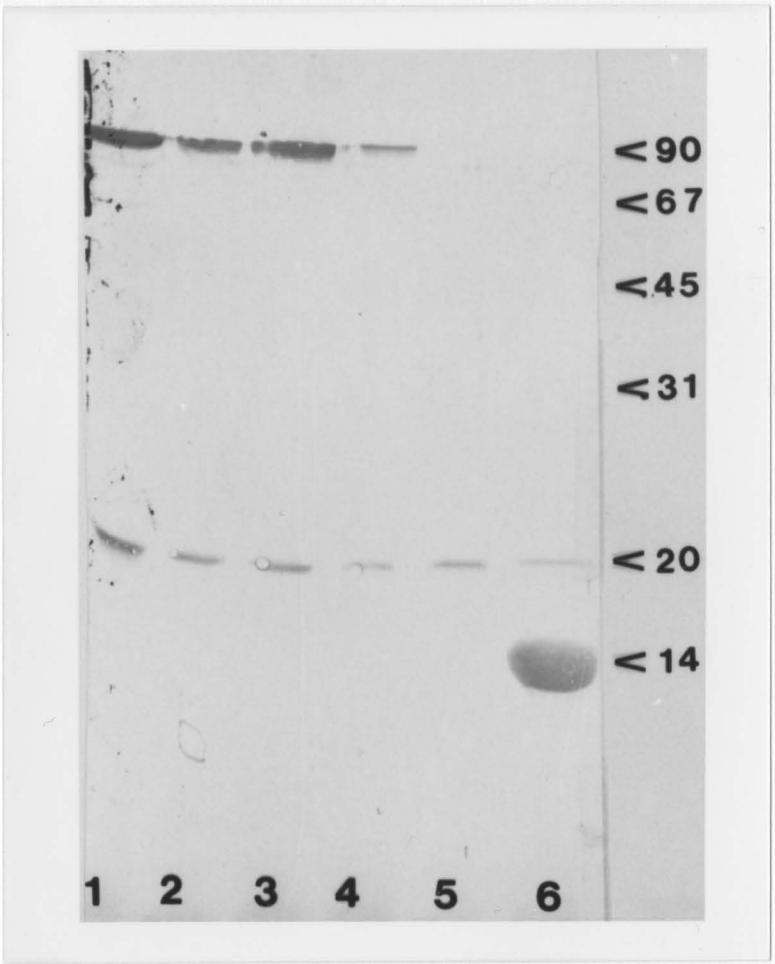

Figure 26. **Antibody to pp20 reacts with 2 proteins that are present throughout development:** Cells were developed on filter discs. At the desired stages the cells on an individual disc were frozen at -80°C. After thawing the cells were scraped from the disc and suspended in 1ml buffer A. The broken cells were then centrifuged at 100,000g for 1hr. Samples of 100,000g supernatants from cells developing at each were subjected to SDS PAGE, western blotting and immunostaining. Lysozyme, added to the purified protein (lane 6) reacts directly with protein A peroxidase and provides a convenient marker. 1. Amoebae 2. Slug stage 3. Culmination stage. 4. Fruit stage 5. Purified pp20 6. Purified pp20 with lysozyme (14.2 KDa)

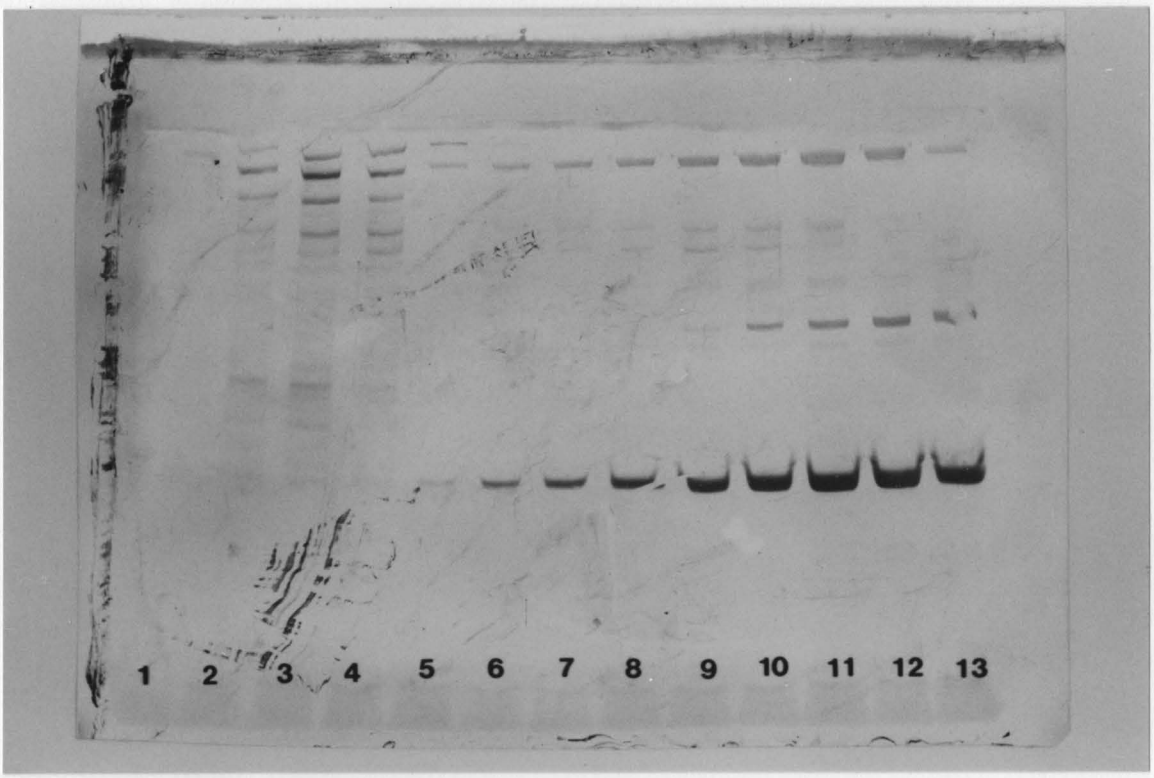

Figure 27. **Relationship between pp20 and pp85 in S300 fractions:** S300 fractions were subjected to SDS PAGE ,western blotting and immunostaining. Lanes 1-13 are fractions 75, 80, 85 etc, respectively.

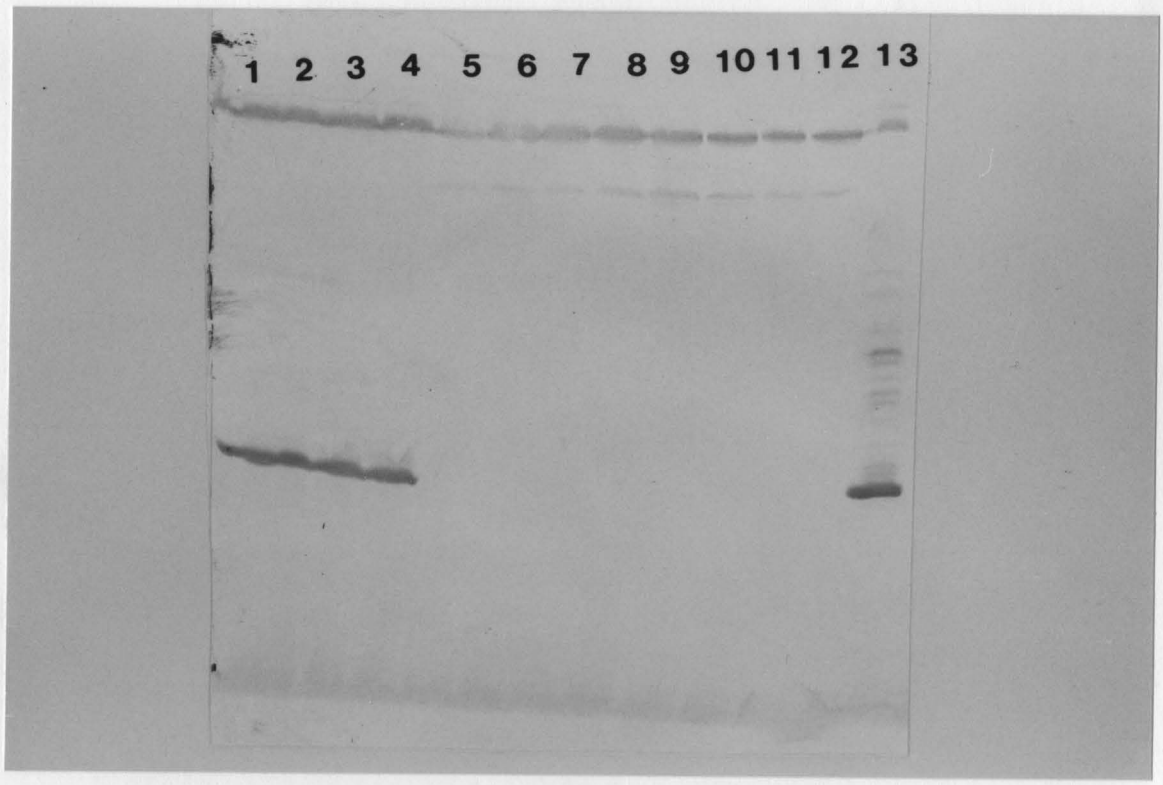

Figure 28. **pp85 in DE52 fractions containing casein kinase activity:** Samples were subjected to SDS PAGE, western blotting and immunostaining. Lanes 1-4, duplicate 100,000g supernatant samples. Lanes 5-12, DE52 gradient fractions containing casein kinase. The peak of casein kinase activity was in fractions loaded on lanes 7-9. Lane 13, DE52 flow-through.

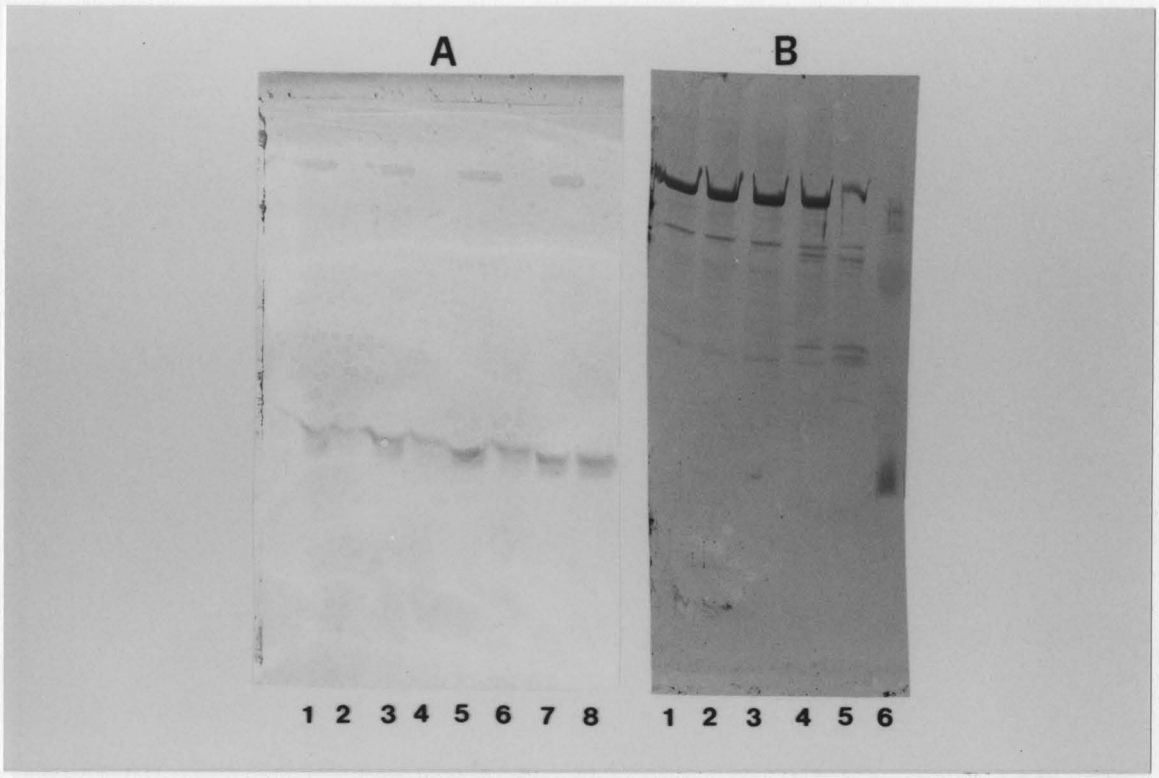

Figure 29. **Partial proteolytic digestions of pp85 in a 100,000g** supernatant and in a casein kinase preparation: The samples were subjected to SDS PAGE (15% gel), western blotting and immunostaining. The sample shown in B was first immunostained (very dark bands) then was stained for protein with amido black (grey bands). A. Aliquots (100µl) of a 100,000g supernatant were incubated without (1,3,5,7) or with (2,4,6,8) 1.4ug B chymotrypsinogen for the following times; Lanes 1-2, 1 sec. Lanes 3-4, 5 min. Lanes 3-4, 10 min. Lanes 5-6, 15 min. Lanes 7-8, 20min. B. Aliquots(100µl) of a casein kinase preparation were incubated 3 min with the following amounts of chymotrypsinogen; 1. none 2. 0.0014ug 3. 0.014ug 4. 0.14ug 5. 1.4ug 6. immunoprecipitated HA F27 not digested.

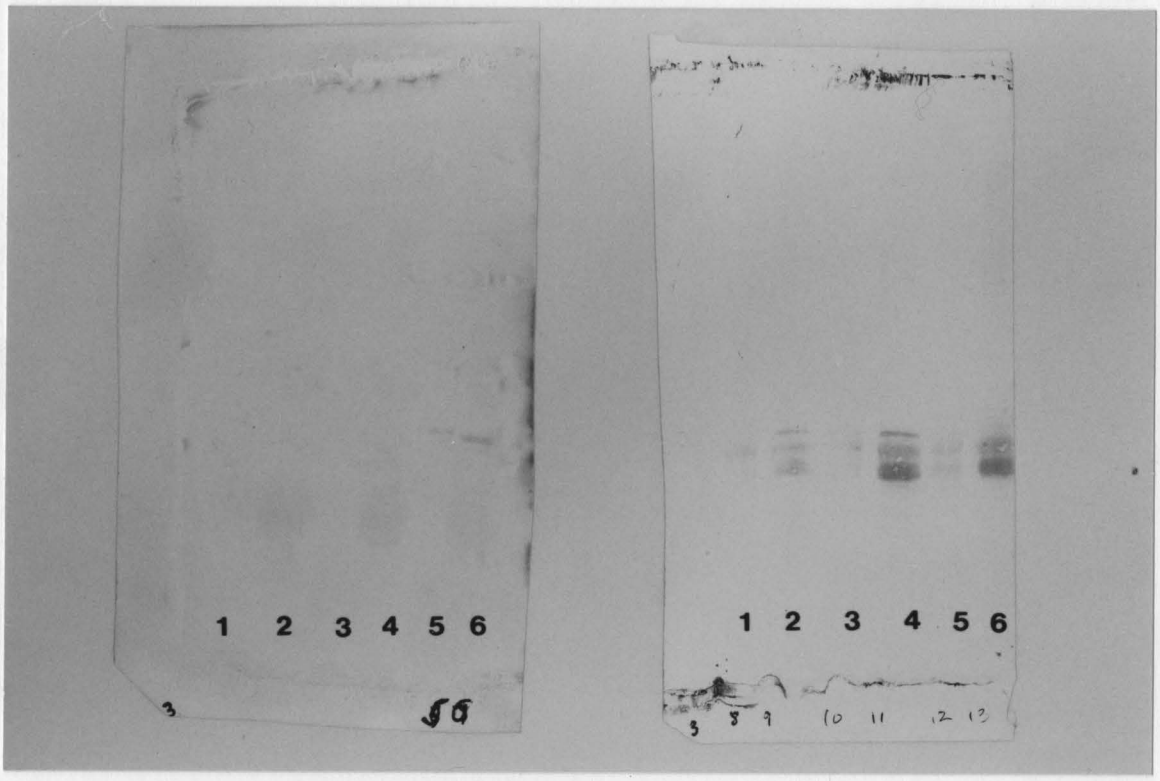

Figure 30. Recognition of similar proteins by an antibody against rabbit phosphorylase kinase and anti pp20/85.: Samples were immunoprecipitated either with anti pp20/85 or with anti rabbit phosphorylase kinase, and then subjected to SDS PAGE (7.5% gel) western blotting and immunostaining with anti rabbit phosphorylase kinase (A) or anti pp20 (B). Lanes 1,3 and 5, are HA F23, casein kinase prep,and rabbit phosphorylase kinase respectively, immunoprecipatated with anti rabbit phosphorylase kinase. Lanes 2,4 and 6 are the same respective samples immunoprecipated with anti pp20/85

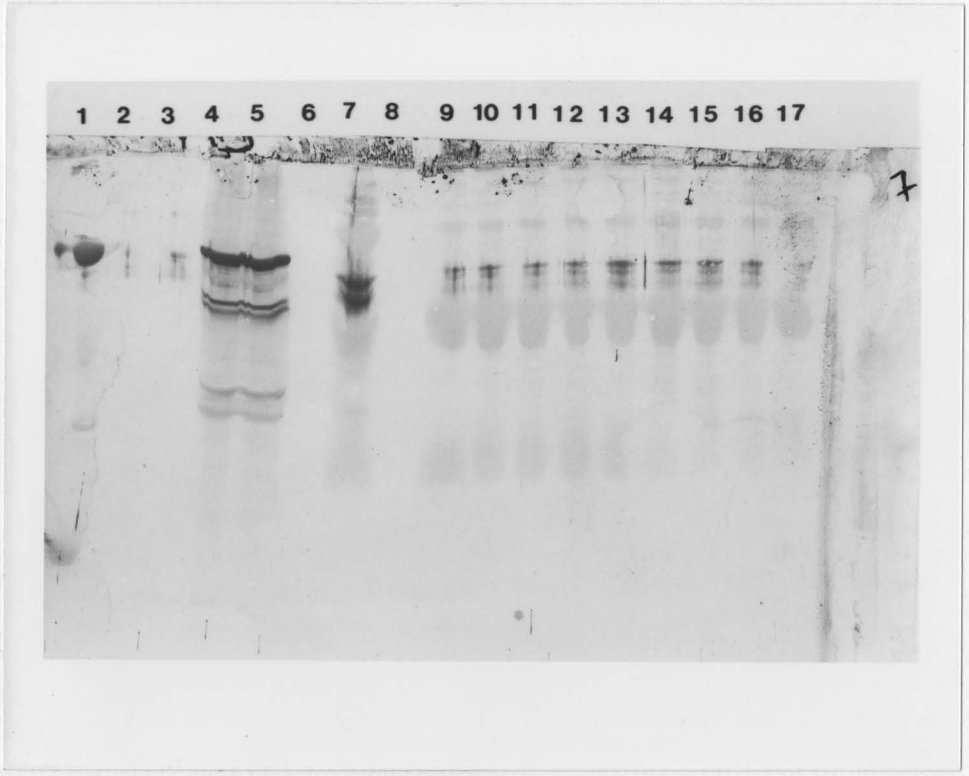

Figure 31. Immunoprecipitation of similar proteins from rabbit phosphorylase kinase by anti pp20/85 and antibody to rabbit phosphorylase kinase.: Samples were subjected to SDS PAGE (15% gel) western blotting and immunostaining with anti pp20/85. After immunostaining (black bands) the blot was stained for protein with amido black (grey bands). The difuse protein staining in the middle region of the blot in lanes 9-17 is due to serum proteins from the immune precipitation. Commercial phosphorylase kinase was loaded on an S300 column. Absorbance at 280nm and phosphorylase kinase activity correlated roughly with each other. Samples extending across the activity peak were immunoprecipitated with antibody against pp20 (lanes 9-17) lane 13 was the peak of activity. Lane 1. A sample of the peak fraction (same as lane 13) was loaded without immunoprecipitation. Lanes 4 & 5. Casein kinase preparation from *Dictyostelium* not immunoprecipitated. Lane 7. Crude rabbit phosphorylase kinase (2mg/ml) not immunoprecipitated.

5.4 *Discussion.*

We have described in this report, a previously undetected protein kinase from *Dictyostelium.* Under our assay conditions pp20 is the major phosphorylated protein in sephacryl 300 fractions and the only phosphorylated protein in hydroxylapatite gradient fractions. It is also the predominant protein in S300 fractions and as such, must represent a relatively major cellular protein. The data presented here is consistent with phosphorylation of pp20 by an intramolecular autophosphorylation reaction. This autophosphorylation may be a regulatory mechanism and in support of this idea we have presented data showing the presence of an endogenous protein phosphatase that recognizes phosphorylated pp20 as a substrate. Thus there exists the potential for regulation by reversible phosphorylation and dephosphorylation. Evidence has been presented that pp20 is in some way related to a higher molecular weight protein, pp85, that is present in the same extracts. The nature of the relationship is presently uncertain. Initially we suspected that pp20 was a proteolytic product of pp85. In fact, we were able to produce a series of lower molecular weight proteins, that were recognized by the antibody, by limited digestion with proteases. However, the observation that in fresh 100,000g supernatants pp80-90 and pp20 are both present, while no related proteins of intermediate size are detected, suggests that pp20 is not a proteolytic product of the 80-90 Da protein. Unless pp20 resulted from a single, precise cleavage of pp85, intermediate sized proteins, such as those observed in S300 fractions, would also be present in the 100,000g extracts. Alternatively, pp85 may represent a tetramer of aggregated pp20. At first this idea seems unlikely since the blots were made from SDS gels run under reducing conditions. On the other hand, when samples of pp20 eluted from a preparative SDS gel were run on a second SDS gel, faint protein staining was sometimes observed at about the same position that pp85 occurs on blots. Thus the possibility exists that pp85 may be a tetramer that is, for some reason, incompletely disrupted by SDS PAGE.

We have shown that anti *Dictyostelium* pp20/85 recognizes a protein present in a commercial preparation of rabbit phosphorylase kinase. The rabbit protein is similar in size to *Dictyostelium*

pp85 as indicated by their migration to about the same position on western blotting. This suggests that *Dictyostelium* pp85 is related to rabbit phosphorylase kinase. This idea is supported by experiments showing that antibody made against rabbit phosphorylase kinase, immunoprecipitates protein from *Dictyostelium* casein kinase extracts. This same casein kinase is also recognized by anti *Dictyostelium* pp20/85.

One explanation of these data, is that pp20 is the catalytic subunit, or part of the catalytic subunit, of phosphorylase kinase from *Dictyostelium.* Alternatively, it might be that anti pp20/85 recognizes common sequences around kinase active sites; but if that is the case then it must be explained why only two bands from 100,000g extracts are immunostained when there are certainly more than two kinases present.

Since pp20 is a major phosphorylated protein, it is curious that it has not been characterized previously. It may be that the inhibition of its autophosphorylating activity by exogenous divalent cations in the absence of EDTA, has allowed pp20 to escape detection. An additional possibility is that the low concentration of ATP used in these experiments (1.6 uM) may encourage autophosphorylation, while the 20-100 fold higher levels used in many kinase reaction mixtures may cause phosphorylation of substrate proteins. This possibility has not yet been tested.

Because phosphorylation and dephosphorylation of proteins is known to be a major intracellular mechanism of regulating growth and development, as well as a variety of other cellular processes, it is important to our understanding of these events that the various enzymes involved be separated, purified and characterized. We have described here the purification and partial characterization of a previously unknown protein kinase from *Dictyostelium* that may be involved in the differentiation of the two cell types. We have shown that an antibody against the purified autophosphorylating 20 KDa protein kinase also recognized an 85KDa *Dictyostelium* protein that we believe possesses casein kinase activity. In addition we have shown that an antibody against rabbit phosphorylase kinase cross reacts with a protein that comigrates on western blotting with the 85 KDa protein immunostained by antibody against the 20KDa *Dictyostelium* protein. Future work will clarify the relationship of the two *Dictyostelium* proteins to each other as well as to rabbit phosphorylase kinase.

Summary

During the course of this project a number of distinct phosphatase and protein kinase activities present in *Dictyostelium* extracts have been resolved and partially characterized. While the physiological functions of these enzymes are unknown, it is not unlikely that some of them play a role in regulating development. At least two of the enzymes described here, the 180 KDa Kemptide phosphatase and the particulate histone phosphatase, vary in activity during development and may themselves be developmentally regulated, while others, such as the 20 KDa autophosphorylating kinase are present in similar amounts throughout development. It is, of course, not necessary for the synthesis of an enzyme that is involved in developmental regulation to be developmentally regulated itself. Control over its activity is all that is required. Such control can be achieved by the timing of synthesis of substrates, by compartmentation of the enzyme or substrates, or by changes in enzyme activity or specificity caused by covalent modifications or associations with other proteins. Thus a key question for future work is how are these particular enzymes regulated? Determining co-factor requirements and optimum conditions for *in vitro* assays is useful but not necessarily indicative of regulatory mechanisms. While the finding that most of these enzymes have subunit structure is suggestive of regulation by association with other proteins, there is little direct data to support that conclusion. We have shown that ethanol treatment of the soluble histone phosphatase both increases its activity and decreases its apparent

molecular weight on gel filtration. Prior to ethanol treatment the histone phosphatase exhibits extreme size heterogeneity. But it is not certain whether the heterogeneity represents different functional forms or merely degradation of an originally homogeneous enzyme. Similarly, the observation that antibody raised against the 20KDa autophosphorylating protein kinase also recognizes pp85 suggests either that aggregation of pp20 occurs or that pp20 is a portion of the larger polypeptide. In either case, a higher molecular weight offers opportunity for more sophisticated regulation.

It has been shown that the pp20 incorporates γ- P^{32} from ATP, apparently by autophosphorylation. This too, may represent regulation, but again it might either be functionally irrelevant or a result of degradation. It could not be determined whether autophosphorylation by the kinase controlled its activity. The reason that it could not, was that the purified enzyme did not utilize any of the exogenous substrates that were tested. That being the case, the only measurement of activity was autophosphorylation itself. The observation that in cruder preparations the enzyme was readily dephosphorylated, while in more purified preparations, no loss of radioactivity occurred, suggested the presence of a protein phosphatase that recognized it as a substrate. Thus there is at least the potential for a control system involving autophosphorylation by the kinase and dephosphorylation by a protein phosphatase.

Of equal importance to the question of regulation, is identification of *in vivo* substrates. I have made the suggestion that the protein phosphatases described in this work function to oppose the action of the cAMP-dependent protein kinase, although if this system is similar to that in mammals, they probably also dephosphorylate a variety of proteins that are not directly acted on by the cAMP-dependent kinase. The unfortunate fact, is that in *Dictyostelium* there are no known *in vivo* substrates for the cAMP-dependent, or any other particular kinase, much less, known substrates for protein phosphatases. That is not to say that phosphorylated proteins haven't been examined, but that the proteins were merely bands or spots on gels, not enzymes of known function.. Clearly, the identification of actual substrates with known functions is vital to any real

understanding of the physiological significance of these enzymes. An obvious step, that is currently feasible is to identify the phosphatase that dephosphorylates the 20 KDa kinase.

Immunologic evidence has been presented which shows that antibody to pp20 cross reacts with a higher molecular weight *Dictyostelium* protein. This higher molecular weight protein, pp85, migrates on SDS PAGE in the vicinity of protein present in commercial rabbit phosphorylase kinase that is also recognized by anti pp20. The case for there being some relationship between rabbit phosphorylase kinase and *Dictyostelium* pp80 is supported by evidence showing that an antibody specifically raised against rabbit phosphorylase kinase, cross reacts with a protein in *Dictyostelium* extracts that comigrates with pp85. The last bit of supporting evidence is that when DE52 fractions containing pp85 and, not coincidently I think, containing casein kinase activity, were mixed with fractions containing phosphorylase "b", phosphorylation of the "b" enzyme could be demonstrated. There are several reasons, however, to doubt that pp85 is part of *Dictyostelium* phosphorylase kinase. First there is the lack of demonstrable inhibition of casein kinase kinase activity by the antibody. This objection is not too serious, however, since the antibody does not inhibit autophosphorylation of pp20 either. More damaging to the case, is the size of the proteins that are recognized. Mammalian phosphorylase kinase is composed of four different types of subunits. The catalytic subunit is a polypeptide of about 45 KDa and that, presumably, would be the subunit anti pp20 would recognize. In fact however the proteins that are jointly recognized by anti pp20 and anti rabbit phosphorylase kinase are 80-90KDa. Furthermore, although the phosphorylase kinase antibody was raised against the holoenzyme (Lawrence et al. 1986), Lawrence did not detect the catalytic subunit in any of their western blotted immune precipitates. It might be argued that the antibodies are recognizing one of the other subunits to phosphorylase kinase but again there are size problems. The alpha and beta subunits are both considerably larger than pp85, at 145 KDa and 125 KDa respectively, while the delta subunit, calmodulin, is only about 17KDa. Thus the significance of the antigenic cross reactivity is uncertain.

The central theme in protein kinase/phosphatase literature has been regulation by phosphorylation/dephosphorylation in response to second messengers elicited by hormonal or

neural stimulation. It has become almost a matter of dogma, that the only mediators of such second messengers are protein kinases and that they exert their influences only through phosphorylation of proteins. This has been accepted even in the face of evidence that the free regulatory subunit of the cAMP-dependent protein kinase may be translocated into the nucleus. The presumption seems to have been, that if the protein kinase did translocate, its function must have been to specifically inhibit the activity of the free catalytic subunit. Recently Jungman and co-workers (Constantinou et al. 1985) reported that the regulatory subunit not only translocates into the nucleus, but that when it is in the phosphorylated state, it functions as a topoisomerase. This finding, if it is confirmed, carries some rather far reaching implications for those studying the intracellular effects of various second messengers.

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