Polyamine-Activated Protein Kinase Reaction from Nuclei and Nucleoli of *Physarum polycephalum* Which Phosphorylates a Unique M_r 70 000 Nonhistone Protein†

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ABSTRACT: Methods are described for the detection and purification of a protein kinase from nuclei and nucleoli of *Physarum polycephalum* which catalyzed transfer of phosphate from [γ-^{32}P]ATP to a unique nonhistone protein of M_r 70 000 in a reaction that was polyamine dependent. Enzymatic phosphorylation of the nonhistone protein by the purified protein kinase was stimulated greatly, at times more than 60-fold, by the polyamines spermidine and spermine. This unique polyamine-dependent reaction was localized on the rDNA minichromosome of the nucleolus. The polyamine-dependent protein kinase, which was first partially purified with the acidic nonhistone protein fraction from isolated nucleoli, was resolved from at least six other protein kinases by phosphocellulose chromatography into a catalytic component of M_r 26 000 and a complex comprised of the catalytic component associated with a phosphate acceptor protein of M_r 70 000. The complex also catalyzed polyamine-dependent phosphorylation of the endogenous M_r 70 000 component. The resolved catalytic component catalyzed polyamine-dependent phosphorylation of a dephosphorylated M_r 70 000 nonhistone protein that had been independently isolated from nucleoli and previously demonstrated to have properties concordant with a specific regulatory role in rRNA gene transcription [Kuehn, G. D., Affolter, H. U., Atmar, V. J., Seebeck, T., Gubler, U., & Braun, R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2541–2545]. These studies indicate one way that the polyamines may regulate rRNA gene transcription through the mediation of a highly specific nonhistone protein kinase.

Phosphorylation of acidic proteins in the nucleus has long been implicated in the regulation of gene expression. Variations in the phosphorylation of the nonhistone chromosomal proteins, in general, have been correlated with elevated rates of RNA synthesis and increased gene expression in numerous biological systems (Kleinsmith, 1974). The function of most of the individual nonhistone proteins, however, remains obscure. Protein kinases (EC 2.7.1.37) localized in the nucleus, which presumably catalyze these modifications, have been previously examined in HeLa S_3 cells (Phillips et al., 1979), beef liver (Kish & Kleinsmith, 1974), and rat liver (Jungmann & Kranias, 1977; Thornburg et al., 1979). Multiple nuclear protein kinases have thus been reported with variable substrate and modifier specificities. Characterization of extensively purified protein kinases of nuclear origin, however, has been reported in only one instance (Thornburg et al., 1979).

We recently identified (Atmar et al., 1978) and characterized (Kuehn et al., 1979) an acidic phosphoprotein of M_r 70 000 from nucleoli of the slime mold *Physarum polycephalum*. This phosphoprotein demonstrated numerous properties concordant with a specific regulatory role in rRNA gene transcription. The phosphoprotein was demonstrated to be a component of a deoxyribonucleoprotein complex isolated from nucleoli that contained palindromic ribosomal deoxyribonucleic acid (rDNA).[1] The purified phosphoprotein bound with high specificity to a region of purified rDNA near the symmetry axis of the rDNA palindrome. It stimulated rRNA synthesis by RNA polymerase I within the deoxyribonucleoprotein complex. Both of these properties were dependent on the phosphorylation state of the nonhistone protein.

Initial interest in the M_r 70 000 nucleolar phosphoprotein derived from our observation that its phosphorylation in intact isolated nuclei or nucleoli was dependent on the polyamines spermidine and spermine (Atmar et al., 1978). This observation suggested the occurrence of a polyamine-dependent protein kinase reaction in these organelles which could utilize the M_r 70 000 nonhistone protein as a highly specific, if not unique (Atmar et al., 1978), substrate for phosphorylation. In this communication, we report methods to identify and isolate a protein kinase from nuclei and nucleoli with these properties.

Experimental Procedures

Organism Culture and Preparation of Crude Extracts. Shake cultures of microplasmodia of *P. polycephalum* strain M_3cV were grown in a medium containing tryptone, yeast extract, and glucose (Chin & Bernstein, 1968). Nuclei were isolated from 48-h shake cultures by the method of Mohberg & Rusch (1971). Nucleoli were isolated from microplasmodia by using the Percoll gradient method (Affolter et al., 1979).

Purified nuclei or nucleoli were ruptured near 5 °C in a prechilled French pressure cell at 44 000 kg/cm^2. All other procedures were also conducted near 5 °C. The extruded homogenate was centrifuged for 15 min at 10000*g*. After the pellet was discarded, the supernatant fraction was dialyzed overnight into 50 mM Tris-HCl, pH 7.5. The resulting crude extract was subsequently assayed or fractionated by column chromatography as described. Column chromatographic media included DEAE-cellulose (Whatman DE52) or phosphocellulose (Sigma, fine mesh, product no. C2258) as indicated.

Protein Kinase Assays. Protein kinase activity was measured by using the filter paper disk procedure (Corbin & Reimann, 1974) with [γ-^{32}P]ATP prepared enzymatically

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate; rDNA, ribosomal deoxyribonucleic acid; rDNP, deoxyribonucleoprotein complex containing rDNA associated with chromosomal proteins; TES, *N*-[tri(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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(Glynn & Chappell, 1964). A mixture of calf thymus histones (Sigma, Type IIA), casein (Wastila et al., 1971), purified M_r 70 000 nonhistone protein from *P. polycephalum* (Kuehn et al., 1979), and a mixture of heterogeneous nonhistone proteins isolated from *P. polycephalum* nuclei were used as potential protein kinase substrates as indicated. The mixture of nonhistone nuclear proteins was prepared by the same procedures that were reported for the isolation of the M_r 70000 nonhistone phosphoprotein through the fractionation step on a column of Bio-Rex 70 (Kuehn et al., 1979). Thereafter, the acidic protein fraction was dialyzed exhaustively against distilled water and was then lyophilized. The lyophilized mixture of nonhistone proteins was reconstituted in water to a concentration of 5 mg/mL. This stock solution was heated in a boiling water bath for 15 min to inactivate endogenous protein kinases and phosphoprotein phosphatases. It was then utilized as a protein substrate in protein kinase reactions.

To test for the phosphorylation of endogenous protein substrates in protein kinase preparations or for autophosphorylation of protein kinase, assays were conducted in the absence of added protein substrate. cAMP, polyamines, sodium molybdate, poly(lysine), poly(arginine), or heat-stable protein kinase inhibitor from rabbit muscle (Sigma) were added when noted.

Protein kinase activity was assayed in a reaction volume of 0.15 mL that contained final concentrations of 60 mM TES, pH 7, with NaOH, 1 mM EGTA, 20 mM KF, 10 mM magnesium acetate, and 1.2 mM [γ-^{32}P]ATP ($\geq 2 \times 10^7$ cpm/μmol as indicated). Protein substrates were added as indicated in the text. The reaction mixture was incubated at 30 °C for 20 min. Assays that contained chromatographically purified protein kinase demonstrated a linear rate of [^{32}P]-phosphate incorporation into the protein substrate over this time interval. Assays which contained crude extracts from nuclei or nucleoli demonstrated deviations in a linear incorporation rate after 10 min of reaction time. The reaction was stopped by addition of 50 μL of ice-cold 50 mg/mL bovine serum albumin containing 100 mM EDTA. A reaction sample of 100 μL was spotted on a 2.5-cm disk of Whatman 3MM paper. Disks were subsequently washed in perchloric acid (Wastila et al., 1971), dried, and counted in 10 mL of scintillation fluid (Kuehn 1974) by liquid scintillation spectrometry.

Isolation and Detection of rDNP. The rDNP complex containing rDNA and RNA polymerase I was isolated by EDTA solubilization of chromatin from isolated nucleoli as described (Seebeck et al., 1979). RNA polymerase I (EC 2.7.7.6) activity in the rDNP complex was assayed in the presence of α-amanitin (Seebeck et al., 1979; Smith & Braun, 1978).

Analytical Methods. Gel electrophoreses were performed by using 8.8% or 15% discontinuous $NaDodSO_4$–polyacrylamide gels (Laemmli, 1970) as modified by Magun (Magun et al., 1975). For determination of zones which contained radioactivity, gels were sectioned into 1-mm transverse slices and were dissolved in 30% H_2O_2 for counting (Magun et al., 1975).

Protein concentrations for preparations of the M_r 70 000 nonhistone protein were determined by the Coomassie brilliant blue dye binding microassay (Schaffner & Weissman, 1973). Other protein solutions were quantitated with Folin's reagent (Lowry et al., 1951).

Results

Stimulation of Protein Kinase Activity by Polyamines in Nuclear and Nucleolar Extracts. We have reported that the polyamines spermidine and spermine stimulated phosphorylation of four major proteins 6- to 30-fold in intact nuclei. Two of these proteins were found localized in the nucleolus (Atmar et al., 1978). Stimulation of protein kinase activity by polyamines could not be demonstrated in crude nuclear extracts. Partial purification of crude nuclear extracts by the method of Kish & Kleinsmith (1974) through Bio-Rex 70 (Bio-Rad, Richmond, CA) treatment did yield preparations which demonstrated polyamine-stimulated protein kinase activity as shown in Table I. However, stimulation was dependent on the protein substrate provided to the assay. Only a nonhistone protein mixture supported polyamine-stimulated activity. Casein and a histone protein mixture did not serve as substrates. Table I also shows that spermidine and spermine stimulated protein kinase activity in crude extracts from nucleoli that had not been fractionated by the Kish and Kleinsmith method. This effect could be demonstrated only when the nonhistone protein mixture served as substrate. Sodium molybdate, a potent inhibitor of endogenous acidic phosphoprotein phosphatase (Stott, 1976; Atmar et al., 1978), was an essential ingredient in these assays in order to consistently observe stimulation by the polyamines.

Table I: Stimulation of Protein Kinase Activity by Polyamines in Nuclear and Nucleolar Preparations[a]

origin of protein kinase preparation[b]	protein substrate[c]	spermidine and spermine addition[d]	[^{32}P]phosphate incorporated[e] (cpm/mg of protein kinase prepn)
nuclear, fractionated	casein	–	39 602
	casein	+	36 041
nuclear, fractionated	histone mixture	–	673
	histone mixture	+	395
nuclear, fractionated	nonhistone mixture	–	8 706
	nonhistone mixture	+	23 495
nucleolar, crude	nonhistone mixture	–	6 084
	nonhistone mixture	+	13 758

[a] For inhibition of endogenous phosphoprotein phosphatase activity, 20 mM Na_2MoO_4 was added to all assays. [b] Procedures for the preparation of nuclear and nucleolar protein kinases were given in the text. The assay protocol was described under Experimental Procedures. [c] The final concentration of protein substrate in the reaction mixture was 2 mg/mL. [d] A combination of 0.5 mM spermidine and 0.5 mM spermine was used in assays where polyamines were added. [e] The specific radioactivity of [γ-^{32}P]-ATP was 5.0×10^7 cpm/μmol for the nuclear kinase assays and 4.30×10^7 cpm/μmol for the nucleolar kinase assays. Values for zero-time control assays have been subtracted from values reported.

DEAE-cellulose Chromatography. Other investigators have reported the presence of multiple protein kinase activities in preparations derived from nuclei (Kish & Kleinsmith, 1974; Dastugue et al., 1974; Takeda et al., 1971; Thornburg et al., 1979) and nucleoli (Olson et al., 1978; Wilson & Ahmed, 1975). A variety of chromatographic media have been used to resolve some of these kinases. However, attempts to isolate a putative polyamine-stimulated protein kinase fraction from *P. polycephalum* in crude extracts of broken nuclei by DEAE-cellulose (Whatman DE52) chromatography with a NaCl gradient (see below) were unsuccessful (data not shown). Only two fractions containing casein kinase activity were isolated. One fraction was cAMP dependent while the second

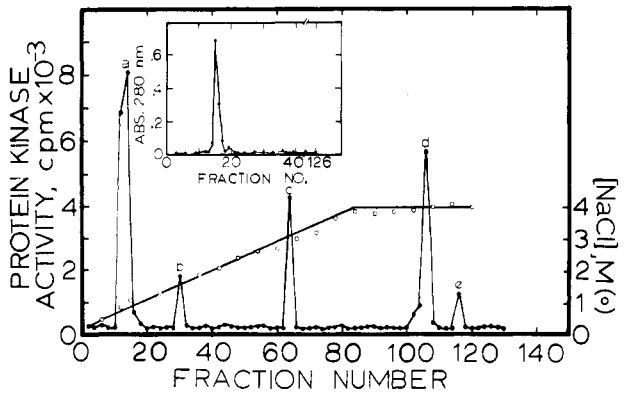

FIGURE 1: DEAE-cellulose (Whatman DE52) column chromatography of protein kinases fractionated from acidic nonhistone proteins previously extracted from isolated nuclei of *Physarum polycephalum*. Partially purified, acidic nonhistone proteins, 5 mg, were applied to a DEAE-cellulose column (2 × 12 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.5 M NaCl. A linear gradient of 400 mL from 0.5 to 4 M NaCl was applied (○). Ten-milliliter fractions were collected. Protein was measured by ultraviolet absorbance at 280 nm (inset). Each fraction was assayed under nine different reaction conditions. Each was tested for protein kinase activity capable of phosphorylating calf thymus histone mixture, casein, or nonhistone protein mixture, all at 2 mg/mL in the assay, in the presence and absence of potential modifiers, 20 μM cAMP or a mixture of spermidine and spermine, 0.5 mM each. Incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP (specific radioactivity 3.51 × 10^8 cpm/μmol) into protein substrates was used to measure protein kinase activity (●). Five distinct protein kinases of variable substrate and modifier specificities were resolved under these conditions. This basic pattern of resolution was repeated in two duplicate experiments.

fraction was cyclic nucleotide independent. Neither of these fractions had the capacity to phosphorylate histone or nonhistone protein substrates. Neither fraction demonstrated phosphorylation of endogenous substrate in their respective preparations.

Marked improvement in the capacity to resolve multiple protein kinases was achieved through preliminary fractionation of nuclei by the method of Kish & Kleinsmith (1974) prior to DEAE-cellulose chromatography. Soluble proteins of the nucleus were first removed from purified nuclei and were treated as previously described through batchwise ion exchange with Bio-Rex 70. The resulting supernatant fraction containing the acidic protein fraction was dialyzed to equivalent conductivity against 50 mM Tris-HCl, pH 7.5, containing 10 mM NaCl. Subsequent application of this fraction to DEAE-cellulose chromatography, as described in Figure 1, yielded five different fractions that exhibited protein kinase activity. Fractions a and c phosphorylated casein. Fractions b, c, and e demonstrated endogenous phosphorylating activity without supply of an exogenous protein substrate but were inactive when exogenous nonhistone protein mixture was provided to the assays. Fractions a and b were cAMP dependent. All other fractions were unaffected by cAMP. Fraction d, which eluted from the column at 4.0 M NaCl, was polyamine dependent in its phosphorylation of components in a nonhistone acidic protein mixture. The addition of 1 mM spermidine, 1 mM spermine, or a combination of 0.5 mM each of spermidine and spermine increased phosphorylation 1.5–4-fold by fraction d. Putrescine did not affect the reaction. No histone kinase activity, either cAMP dependent or cAMP independent, was detected in any fractions derived from chromatography on DEAE-cellulose.

Phosphocellulose Chromatography of Nuclear Preparations. An unusually high multiplicity of protein kinases has been reported in beef liver nuclei fractionated by phosphocellulose chromatography (Kish & Kleinsmith, 1974). For determination of whether a comparable degree of fractionation could be attained from nuclei of *P. polycephalum*, the nonhistone chromatin phosphoprotein fraction was prepared by the method of Kish & Kleinsmith (1974) through treatment with Bio-Rex 70. The acidic protein fraction was then chromatographed on phosphocellulose as described in Figure 2A. All fractions were tested for their capacity to phosphorylate casein, calf thymus histone mixture, and nonhistone mixture from *P. polycephalum*. Seven different fractions containing protein kinase activity were isolated. Fractions a, b, and f phosphorylated casein. Of these, only fraction a was cAMP dependent. Fraction b was variable and appeared in only two of four isolation trials. Fractions c and d were histone kinases that were unaffected by cAMP. A mixture of spermine and spermidine, 0.5 mM each, inhibited fraction c approximately 25%. Fractions b, e, f, and g could phosphorylate nonhistone proteins from *P. polycephalum*. Endogenous phosphorylation was exhibited by fractions a, f, and g in the absence of protein substrates supplied exogenously. Many protein kinases are autophosphorylated (Rubin & Rosen, 1975; Thornburg et al., 1979). These results indicated that fractions a, f, and g contained protein kinases which underwent autophosphorylation or that they cochromatographed with other proteins which served as substrates. Fraction e contained polyamine-stimulated protein kinase activity and phosphorylated only nonhistone protein substrate. Spermidine and spermine, 0.5 mM each, increased phosphorylation 3-fold. This polyamine-stimulated fraction has been prepared without a failure in eight different fractionation experiments in our laboratory. cAMP-dependent nonhistone protein kinase activity was found in fraction g.

Phosphocellulose Chromatography of Nucleolar Preparations. This laboratory has recently reported marked stimulation of nonhistone protein phosphorylation in isolated nucleoli by the polyamines spermidine and spermine. Moreover, one major protein substrate of M_r 70 000 in nucleoli was phosphorylated in vivo by a polyamine-dependent protein kinase reaction. This phosphoprotein markedly enhanced rRNA gene transcription in nucleolar chromatin (Kuehn et al., 1979). Thus, it was of major importance to attempt to demonstrate localization of the nuclear polyamine-stimulated protein kinase within the nucleolus. Soluble proteins from purified nucleoli were prefractionated through batchwise treatment with Bio-Rex 70 and dialysis as described for nuclear proteins in the previous section. The acidic nonhistone protein fraction was subsequently chromatographed on a column of phosphocellulose. Figure 2B shows the gradient elution profile. All fractions were assayed for their capacity to phosphorylate nonhistone protein mixture in the presence and absence of a combination of 0.5 mM spermidine and 0.5 mM spermine. Indeed, fraction b demonstrated nearly an absolute dependence on the polyamines to catalyze phosphorylation. The activity eluted reproducibly from phosphocellulose at 0.7 M NaCl in seven isolation trials. Storage of this fraction at 3 °C in 50 mM Tris-HCl, pH 7.5, and 0.1 M NaCl preserved the enzymatic activity for several weeks.

Resolution of Catalytic Component from Phosphate Acceptor Protein. Fraction b in Figure 2B also exhibited polyamine-dependent, endogenous phosphorylation capacity in the absence of exogenous nonhistone protein mixture. Fraction a contained slight nonhistone protein kinase activity but no endogenous phosphorylation capacity. The resolution of fractions a and b was observed in repeated isolation trials. This indicated the possibility that a catalytic component and a phosphate acceptor protein cochromatographed in fraction b

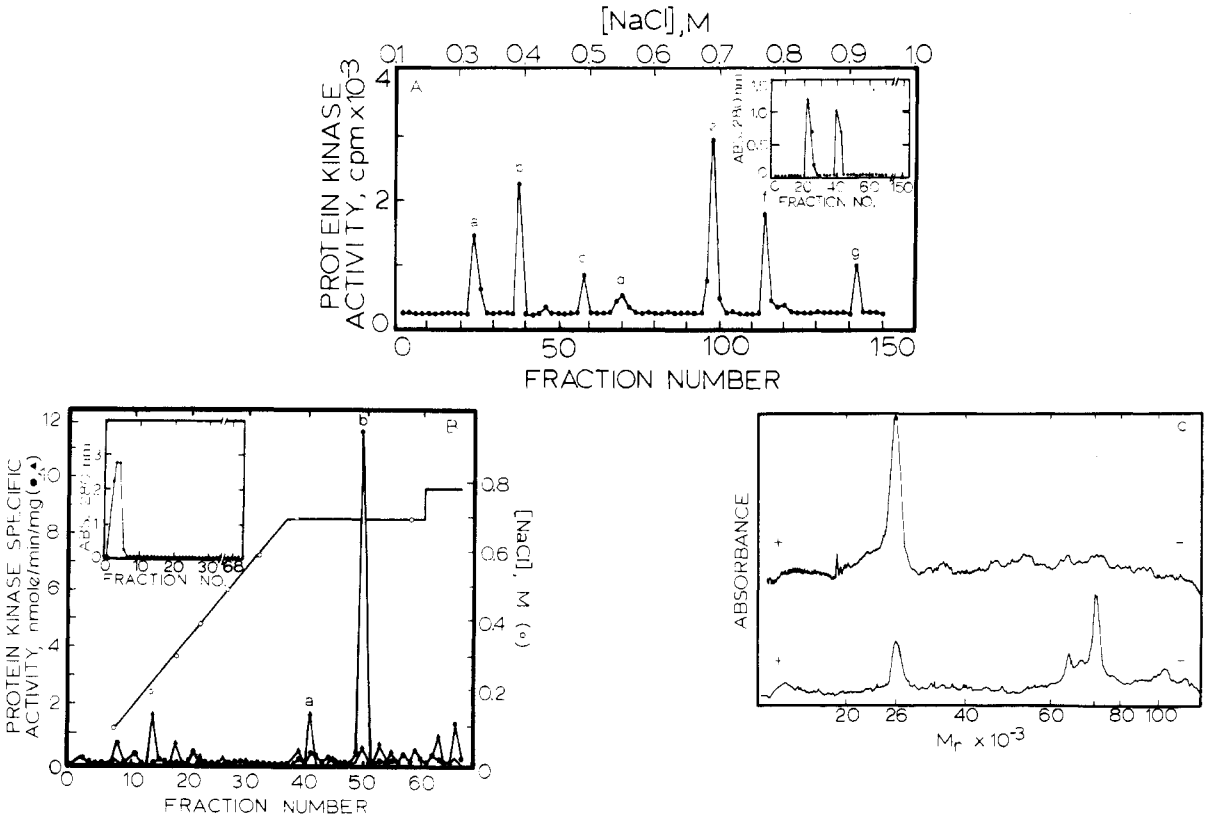

FIGURE 2: (A) Phosphocellulose column chromatography of protein kinases fractionated from acidic nonhistone proteins extracted from isolated nuclei of *Physarum polycephalum*. Partially purified, acidic nonhistone proteins, 14 mg, were applied to a phosphocellulose column (0.9 × 15 cm) equilibrated with 50 mM Tris-HCl, pH 7.5. Proteins were fractionated by stepwise gradient elution with 30-mL increments of 0.1 M NaCl. Fractions of 15 mL were collected. Each fraction was tested for protein kinase activity capable of phosphorylating endogenous protein substrates concurrently in the fraction, casein, calf thymus histone mixture, or a nonhistone protein mixture from nuclei of *P. polycephalum* in the presence or absence of 20 μM cAMP or a mixture of spermidine and spermine, 0.5 mM each. Protein was measured by ultraviolet absorbance at 280 nm (inset). Incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP (specific radioactivity 3.97 × 10^8 cpm/μmol) into protein substrates was used to measure protein kinase activity (●). Under these conditions, seven distinct fractions with protein kinase activity could be resolved. With the exception of variable detection of peak b, this pattern of resolution was repeated in four separate experiments. (B) Phosphocellulose column chromatography of protein kinase fractionated from acidic nonhistone proteins extracted from nucleoli of *Physarum polycephalum*. Partially purified, acidic nonhistone proteins, 45 mg, were applied to a phosphocellulose column (1.1 × 12 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl. Proteins were fractionated with a 300-mL linear gradient from 0.1 to 1 M NaCl (○). Fractions of 5.2 mL were collected. Each fraction was tested for protein kinase activity capable of phosphorylating components in a nonhistone protein mixture prepared from nuclei of *P. polycephalum*. Assays were conducted in the presence (●) or absence (▲) of the polyamines spermidine and spermine, 0.5 mM each. Incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP (specific radioactivity 4.06 × 10^7 cpm/μmol) into protein substrates was used to measure protein kinase activity. Protein was measured by ultraviolet absorbance at 280 nm (inset). Under these conditions, a major fraction containing polyamine-dependent protein kinase activity could be resolved. (C) Densitometer tracings of polyacrylamide gel–$NaDodSO_4$ electrophoresis patterns of fraction a (upper tracing) and fraction b (lower tracing) from Figure 2B. Approximately 20 μg of protein from each fraction was applied to a 15% polyacrylamide–$NaDodSO_4$ gel. Electrophoresis was carried out at 2 mA per gel tube for approximately 7 h. The gels were stained with 0.1% Coomassie brilliant blue reagent in 9% acetic acid and 50% methanol and then destained by horizontal diffusion in 7.5% acetic acid and 5% methanol at room temperature. The stained gels were scanned at 595 nm in a spectrometer equipped with a Gilford linear transport accessory. Molecular weights, indicated on the lower axis, were established with marker proteins.

and that fraction a contained only the catalytic component resolved from the acceptor protein.

To test this proposal, it was first necessary to determine which nuclear and nucleolar protein(s) served as [^{32}P]phosphate acceptor. When protein kinase samples from fraction e (Figure 2A) or fraction b (Figure 2B) were incubated with the nuclear nonhistone protein mixture and [γ-^{32}P]ATP, only one major protein band was labeled in a polyamine-dependent reaction as shown by $NaDodSO_4$ gel electrophoresis in Figure 3. The predominant phosphate acceptor was a polypeptide of M_r 70000. A significantly lesser amount of [^{32}P]phosphate was incorporated into a peptide of M_r 130000. Moreover, $NaDodSO_4$ gel electrophoresis of samples from fraction b (Figure 2B) which were subsequently stained with Coomassie blue to reveal protein zones yielded protein bands at M_r 26000 and 70000 (Figure 2C). Similar gels of samples from fraction a (Figure 2B) yielded only a single band of M_r 26000 as shown in Figure 2C.

These results strongly indicated that the M_r 70000 protein phosphorylated in the nonhistone protein mixture by fraction b (Figure 2B) was the same protein that was previously purified and found to stimulate rRNA gene transcription in vitro (Kuehn et al., 1979). A protein kinase of M_r 26000 resolved in fraction a (Figure 2B) appeared to be the catalytic component responsible for the phosphorylation. So that this proposal could be tested, purified and dephosphorylated M_r 70000 nonhistone protein isolated from nucleoli (Kuehn et al., 1979) was incubated with protein kinase from fraction a and [γ-^{32}P]ATP. Table II shows that the protein kinase activity was totally dependent on the addition of polyamines and the M_r 70000 nonhistone protein substrate.

NaCl Dependence of Polyamine Activation. Sodium chloride was a component at 48 mM concentration in all assays that contained the polyamine-dependent protein kinase prepared from phosphocellulose chromatography. The effects of NaCl on the enzyme activity and on polyamine activation were

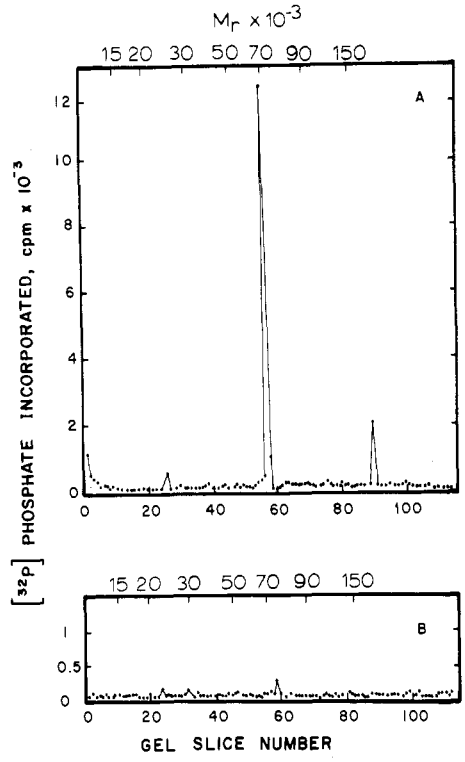

FIGURE 3: Correspondence of protein and [^{32}P]phosphate in NaDodSO$_4$–polyacrylamide rod gels following labeling of a heterogeneous nonhistone protein mixture with [γ-^{32}P]ATP by purified polyamine-dependent protein kinase in an in vitro protein kinase assay. (A) The reaction mixture in 0.15 mL contained 60 mM TES, pH 7, with NaOH, 1 mM EGTA, 10 mM magnesium acetate, 1.2 mM [γ-^{32}P]ATP (specific radioactivity 3.66 × 10^8 cpm/μmol), 0.33 mM each of spermidine and spermine, 2 mg/mL nonhistone protein mixture, and 4.5 μg of purified protein kinase (fraction a, Figure 2B). The reaction was incubated at 30 °C for 20 min and then stopped by addition of 1% NaDodSO$_4$, 65 mM Tris-HCl, pH 7.5, and 5% β-mercaptoethanol. The mixture was heated in a boiling water bath for 2 min prior to application onto an 8.8% polyacrylamide gel. After electrophoresis, the gel was subsequently sliced into 1-mm transverse sections that were dissolved in 30% H_2O_2 for scintillation spectrometry. (B) The protocol was the same as for panel A, except that spermidine and spermine were omitted from the reaction mixture.

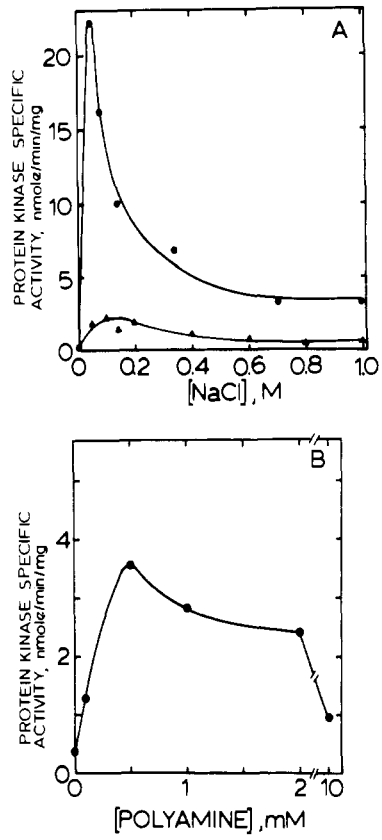

FIGURE 4: (A) Effect of NaCl concentration on nucleolar polyamine-dependent protein kinase activity. Samples of protein kinase from fraction b, Figure 2B, were dialyzed against 50 mM Tris-HCl, pH 7.5, and 50 mM $MgCl_2$ to remove NaCl as determined by conductivity measurement. Assays were conducted in the absence (▲) or the presence (●) of the polyamines, spermidine and spermine, 0.5 mM each, NaCl as indicated, and other components given under Experimental Procedures. Incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP (specific radioactivity 4.91 × 10^7 cpm/μmol) into endogenous nonhistone protein (2 μg/mL) was used to determine protein kinase activity. (B) Effect of polyamine concentration on nucleolar polyamine-dependent protein kinase activity. Samples of protein kinase from fraction b, Figure 2B, were dialyzed against 50 mM Tris-HCl, pH 7.5, and 10 mM NaCl. Reaction mixtures contained 50 mM NaCl, other components given under Experimental Procedures, and a combination of equimolar amounts of spermidine and spermine to yield the varied total polyamine concentrations indicated. Incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP (specific radioactivity 4.38 × 10^7 cpm/μMol) into endogenous nonhistone protein (11 μg/mL) was used to determine protein kinase activity.

therefore studied. The apparent capacity of the polyamines to activate the protein kinase was strongly dependent on NaCl concentration. A narrowly defined concentration of NaCl in the region of 50 mM was essential for optimum activation by spermidine and spermine (Figure 4A). Polyamines failed to activate in the total absence of NaCl. Increasing concentrations of NaCl greater than 50 mM progressively abolished the polyamine activation (Figure 4A). Importantly, NaCl alone failed to mimic the activation phenomenon of the polyamines (Figure 4A,B). The optimum total polyamine concentration required to fully activate the protein kinase at a fixed NaCl concentration of 50 mM was 0.5 mM (Figure 4B). Approximately 80% of full activation was achieved at 1 mM total polyamines and 50 mM NaCl (Figure 4B). The effects of other univalent salts other than NaCl have not been investigated.

Localization of Polyamine-Dependent Protein Kinase Reaction on rDNP. Previously we reported that the M_r 70000 phosphoprotein could be found associated with an rDNP complex that contained rDNA in nucleoli. Its phosphorylation in intact nucleoli with [γ-^{32}P]ATP was polyamine dependent (Kuehn et al., 1979). The finding that nucleoli also contained a polyamine-dependent protein kinase that catalyzed phosphorylation of the M_r 70000 nonhistone protein and that the two copurified as a complex raised the question of whether the kinase might also be associated with the rDNP complex. Figure 5 shows that this is indeed the case. Polyamine-dependent protein kinase activity cosedimented with the rDNP minichromosome during sucrose gradient density centrifugation. Under the conditions of the protein kinase assay, no protein kinase activity that was determined in the absence of the polyamines was observed to be associated with the rDNA minichromosome. A polyamine-independent protein kinase activity was observed in a fraction that sedimented more rapidly than the nucleolar rDNP complex. We attribute this to possible contamination of the preparation with nucleoplasmic chromatin.

Potential Effectors of Polyamine-Dependent Protein Kinase Reaction. Polycations other than polyamines have been reported to alter the catalytic capacity of certain protein kinases (Thornburg et al., 1979; Criss et al., 1978a; Yamamoto et al., 1979). Table III shows the relative effectiveness of several basic proteins on the phosphorylation of endogenous M_r 70000 nonhistone protein. None of these basic proteins could serve as phosphate acceptor. Poly(lysine) and poly(arginine) could

Table II: Demonstration That a M_r 70 000 Nonhistone Protein Is a Substrate for Nucleolar Polyamine-Dependent Protein Kinase Resolved by Phosphocellulose Chromatography

addition to enzyme reaction lacking phosphate-acceptor protein substrates[a]	protein kinase sp act.[d] [pmol min^{-1} (mg of enzyme)$^{-1}$]
none	0
polyamines[b]	0.7
M_r 70 000 nonhistone protein (1.3 μg)	14
M_r 70 000 nonhistone protein (6.5 μg)	11
polyamines + 70 000 M_r nonhistone protein (1.3 μg)[c]	70
polyamines + M_r 70 000 nonhistone protein (6.5 μg)[c]	130

[a] Protein kinase (2.33 μg) from fraction a, Figure 2B, was used in each assay. The assay components contained TES buffer, EGTA, KF, magnesium acetate, and [γ-^{32}P]ATP (specific activity 4.22×10^9 cpm/μmol) at concentrations indicated under Experimental Procedures. [b] Polyamines were a mixture of spermidine and spermine, 0.5 mM each. [c] These data were taken from a double-reciprocal replot of a curve for rate [pmol min^{-1} (mg of enzyme)$^{-1}$] vs. concentration of M_r 70 000 nonhistone protein substrate (μg mL^{-1}). The double-reciprocal plot yielded a V_m of 166 pmol min^{-1} (mg of enzyme)$^{-1}$ and a K_m of 18 μg mL^{-1}. [d] Values for zero-time control assays have been subtracted from values reported.

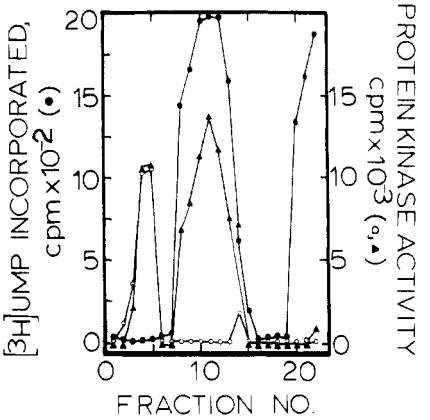

FIGURE 5: Cosedimentation of a deoxyribonucleoprotein particle containing rDNA and polyamine-dependent protein kinase activity. Deoxyribonucleoprotein derived from lysed nucleoli was sedimented through 5 mL of a 15–40% sucrose gradient in a SW65 Beckman rotor at 40 000 rpm and 2 °C for 90 min. The contents of the centrifuge tube was then fractionated into 0.23-mL fractions by pumping out the gradient from the bottom. A 10-μL sample from each fraction was assayed for RNA polymerase activity (●) in the presence of 1 μg/mL of α-amanitin. A 55-μL sample from each fraction was also assayed for polyamine-dependent protein kinase activity by phosphorylation of endogenous protein substrate in the presence (▲) and absence (○) of 0.5 mM each of spermidine and spermine. The bottom of the gradient tube is shown to the left.

partially mimic the activation effect of polyamines. Cyclic nucleotides cAMP and cGMP had no appreciable effect on the purified nuclear protein kinase in the presence or the absence of the polyamines. Heat-stable protein kinase inhibitors isolated from rabbit muscle and from *P. polycephalum*, which were found to inhibit cAMP-dependent protein kinases from the respective host organism, did not inhibit the polyamine-dependent protein kinase reaction.

Discussion

Our findings demonstrate that at least seven distinct protein kinase activities can be fractionated from nuclei of *Physarum polycephalum* by chromatography on phosphocellulose. Resolution of these activities on phosphocellulose required preliminary isolation of the bulk nonhistone acidic protein fraction by the method of Kish & Kleinsmith (1974). Although it cannot be equivocally concluded that these distinct fractions represent unique enzymes, it would appear that the apparent heterogeneity is authentic. Each of the seven protein kinases exhibited differential specificities for protein substrates such as histones, casein, nonhistone proteins, or endogenous substrates. Selected fractions responded discriminantly toward the catalytic modifiers cAMP or the polyamines spermidine and spermine.

Table III: Effect of Polyamines, Other Polycations, Cyclic Nucleotides, and Heat-Stable Protein Kinase Inhibitors on the Activity of Purified Polyamine-Dependent Protein Kinase from Nuclei of *Physarum polycephalum*

protein kinase prepn[a]	addition[b]	protein kinase sp act.[c] [pmol min^{-1} (mg of enzymes)$^{-1}$]
1	none	65
	polyamines	301
	poly(lysine)	136
	poly(arginine)	102
	cAMP	64
	cGMP	70
	polyamines + cAMP	291
	polyamines + cGMP	268
2	none	9
	polyamines	105
	heat-stable inhibitor (rabbit)	6
	polyamines + heat-stable inhibitor (rabbit)	96
	heat-stable inhibitor (*Physarum*)	4
	polyamines + heat-stable inhibitor (*Physarum*)	107

[a] The protein kinase preparations were purified by chromatography on phosphocellulose and correspond to fraction b, Figure 2B. [b] Concentrations were the following: polyamines spermidine and spermine at 0.5 mM each; poly(lysine) or poly(arginine) at 200 μg/mL; cAMP or cGMP at 20 μM each; 0.33 mg/mL heat-stable protein kinase inhibitor from rabbit or *Physarum*. [c] The specific radioactivity of [γ-^{32}P]ATP was 2.82×10^8 cpm/μmol.

Of primary importance to the objectives of this study was the discovery of the polyamine-dependent protein kinase enzyme that could be purified from isolated nuclei or nucleoli and the identification of its naturally occurring phosphate acceptor protein. The natural protein substrates for cyclic nucleotide independent protein kinases from the nucleus have not heretofore been categorized, and little information is available regarding the physiological roles or properties of these proteins. The experimental results described in this paper show that a portion of the nucleolar polyamine-dependent protein kinase is associated with its substrate protein after chromatography on phosphocellulose. Phosphorylation of the substrate protein demonstrated an absolute requirement for spermidine and spermine. The substrate protein that copurified with the catalytic component had an apparent monomer M_r of 70 000. The phosphate acceptor protein requirement was also met by a M_r 70 000 nonhistone acidic protein previously purified in this laboratory from nuclei or nucleoli of *P. polycephalum*. This nonhistone protein demonstrated numerous properties in vitro, suggesting that it may specifically regulate functions of nucleolar rDNA in a manner dependent on its degree of phosphorylation: it was a component of a transcriptionally active deoxyribonucleoprotein complex composed of nucleolar RNA polymerase I, rDNA, and a complement of uncharacterized proteins; it specifically bound to a defined region of the palindromic, satellite rDNA from *P. polycephalum*; and

it stimulated transcription only of rRNA gene regions in vitro by isolated rDNA chromatin.

The apparent M_r of the catalytic component of the polyamine-dependent protein kinase reaction was 26 000. A fraction from phosphocellulose chromatography which eluted just prior to the apparent kinase–acceptor protein complex also contained a polypeptide of M_r 26 000. This protein kinase was shown to phosphorylate exogenous, purified M_r 70 000 nonhistone protein in a polyamine-dependent reaction. We interpret these collective results as indicative of resolution of the catalytic component of M_r 26 000 from the phosphate acceptor protein of M_r 70 000. The natural phosphate acceptor protein is apparently the same nonhistone phosphoprotein that was identified (Atmar et al., 1978) and purified (Kuehn et al., 1979) by us earlier. This claim is supported by our earlier observations that the M_r 70 000 phosphoprotein was associated with the rDNA minichromosome in nucleoli and that it was the only nonhistone protein phosphorylated at that site in intact nucleoli by a polyamine-dependent reaction (Kuehn et al., 1979). The present studies now demonstrate copurification of a polyamine-dependent protein kinase–acceptor protein complex and that the polyamine-dependent protein kinase is also a component of the rDNA minichromosome. These intimate associations observed among the kinase, the acceptor protein, and the rDNA minichromosome hint at a vital but uncharacterized role for the polyamines in the control of rRNA gene expression. Conclusive identity of the phosphate acceptor protein with the previously purified M_r 70 000 nonhistone protein must await the task of its isolation and determination of its amino acid composition.

These results are reminiscent of recent work by Criss and co-workers on the isolation and resolution of the catalytic component and phosphate acceptor protein of a protein kinase from Morris hepatoma 3924A (Criss et al., 1978a,b; Yamamoto et al., 1979). From this source, a cyclic nucleotide independent protein kinase of M_r 26 000 and a phosphate acceptor protein of M_r 70 000 copurified through two ion-exchange columns and isoelectrofocusing treatment. The protein kinase was found at elevated levels in numerous tumor tissues. However, unlike the polyamine-dependent protein kinase from *P. polycephalum*, that from hepatoma was of cytoplasmic origin and was stimulated as much as 10-fold by polycations such as histones, poly(lysine), or poly(arginine). Moreover, the kinase utilized casein as substrate. A cyclic nucleotide independent protein kinase from rat liver nuclei has recently been isolated with an M_r of 28 000. This kinase preferred acidic protein substrates but demonstrated no strict requirement for the polyamines (Thornburg et al., 1979).

The term "polyamine-dependent protein kinase" has been used in this communication only in reference to the requirement for spermidine and spermine for phosphorylation of the M_r 70 000 nonhistone protein substrate. This term is not meant to imply direct interaction with and activation of the protein kinase itself. Nothing in the present report identifies the actual site of action of the polyamines which confers substrate properties to the M_r 70 000 protein. However, preliminary interpretation of incipient experiments hints that the polyamines primarily affect the protein substrate as opposed to the kinase itself (Yamamoto et al., 1979; Ahmed et al., 1978). First, the maximum rate and final extent of phosphorylation of the polyamine-activated reaction vary with the amount of protein substrate used, thus giving the impression that increased complexation occurs between polyamines and the phosphate acceptor protein. Second, the polyamines do not activate the protein kinase reaction with any other substrate except the M_r 70 000 nonhistone protein. It should also be emphasized that the activation effect by polyamines is not due to nonspecific changes in the ionic strength or to the substitution of spermidine and spermine for magnesium ion since maximal stimulation by 1 mM polyamines is observed only at optimal (>0.5 mM) magnesium ion concentrations (Atmar et al., 1978). Although NaCl is essential for activation by the polyamines, it cannot substitute for these nitrogenous cations. Conclusive identification of the site of interaction for the polyamines will require construction of appropriate polyamine analogues with the capacity to form irreversible complexes with the protein substrate or the protein kinase. These are now being synthesized in our laboratory.

The polyamine-dependent property of the protein kinase reaction varied from approximately 3-fold (Table III) to 60-fold (Figure 3) stimulation as compared to control assays lacking polyamines. Essentially absolute dependency of the kinase reaction could be demonstrated in some cases (Figure 5). We have not yet been able to determine the cause of this variability. It may be related to variations in the phosphorylation state of the M_r 70 000 substrate used which has multiple phosphorylatable sites (Atmar et al., 1978; Kuehn et al., 1979). Alternatively, additional chemical modifications of the M_r 70 000 substrate apart from phosphorylation may determine its capacity to serve as a protein kinase substrate. Continued studies by us on the composition of this nonhistone protein have indicated that it contains a component that is released on acid hydrolysis which is more basic that the amino acid arginine. All [^{32}P]phosphate which was incorporated into the M_r 70 000 substrate by the polyamine-dependent protein kinase could be hydrolyzed to free [^{32}P]phosphate with alkaline phosphatase (Kuehn, 1979). This suggested that the phosphorylatable sites consisted of serine or threonine residues and that the unidentified component released on acid hydrolysis is not a product of protein kinase action.

Attempts to chemically characterize the nonhistone chromosomal proteins have been impeded for lack of specific methods to prepare their native, chemically modified forms and for lack of an assay to judge their functional properties. The phosphorylated form of the M_r 70 000 nonhistone protein that we have previously characterized demonstrates properties in vitro that are indicative of exerting a regulatory role on rRNA gene transcription in *P. polycephalum*. Heretofore, we have relied on in vivo phosphorylation of the protein in intact isolated nuclei or nucleoli prior to its purification. The isolation of the highly specific polyamine-dependent protein kinase makes it feasible to prepare the phosphorylated form of the M_r 70 000 nonhistone protein in vitro. Procedures for dephosphorylating this nonhistone protein have been described (Kuehn et al., 1979). Methods are now available to characterize the functional properties of this chromosomal protein in its phospho and dephospho forms.

Added in Proof

Since this paper was submitted for publication, V.J.A. and G.D.K. have found purified preparations of the M_r 70 000 nonhistone protein to contain ornithine decarboxylase (EC 4.1.1.17) activity. Conversely, purified ornithine decarboxylase, prepared by published procedures, is an effective substrate for the polyamine-dependent protein kinase. Thus, we propose that the unique protein substrate for the polyamine-dependent protein kinase reported in this paper is the enzyme, ornithine decarboxylase.

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Single Subunits of Sepharose-Bound Pyruvate Kinase Are Inactive†

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ABSTRACT: Bovine skeletal muscle pyruvate kinase was covalently coupled to Sepharose that had previously been activated by low concentrations of cyanogen bromide. Reaction conditions were chosen such that essentially all tetrameric enzyme molecules were covalently bound via a single subunit. Denaturation of the immobilized enzyme with guanidine hydrochloride followed by removal of noncovalently bound subunits and denaturant resulted in essentially no enzymatic activity remaining bound to the resin. Thus, single immobilized subunits of bovine pyruvate kinase were inactive. Sepharose-bound enzymatic activity could be recovered by adding soluble renaturing enzyme subunits to the immobilized monomers. The former combine noncovalently with the latter, presumably resulting in re-formation of bound tetramers, and an average recovery of 61% of the original matrix-bound activity was observed. While interactions with other enzyme subunits appear to be necessary for catalytic activity of bovine muscle pyruvate kinase, these subunit interactions apparently can be provided by chemically modified subunits. Soluble, renaturing subunits from enzyme that had been inactivated by treatment with trinitrobenzenesulfonic acid were able to interact with matrix-bound single subunits, thereby restoring the enzymatic activity of the latter.

Bovine muscle pyruvate kinase (EC 2.7.1.40) consists of four subunits of 57 000 daltons each (Cardenas et al., 1973). Four phosphoenolpyruvate binding sites occur per tetramer.

Pyruvate kinase can be reversibly renatured after its denaturation in solutions of urea or guanidine hydrochloride (Johnson et al., 1969; Cottam et al., 1969; Cardenas & Dyson, 1973; Strandholm et al., 1976; Cardenas et al., 1977). Recovery of 65–90% of the original enzymatic activity occurs when the urea or guanidine-HCl is removed, via either dilution or dialysis, and the enzyme is incubated under mild conditions in the presence of mercaptoethanol or dithiothreitol.

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