Purification and Characterization of Novikoff Ascites Tumor Protein Kinase†

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ABSTRACT: A protein kinase, designated KII, has been purified 5000-fold from Novikoff ascites tumor cells. The purification procedure also allows for the purification of a second major protein kinase, designated KI, as well as RNA polymerase I and II. Purified KII has a sedimentation constant of 7.6 S and a Stokes radius of 39 Å, suggesting a molecular weight of about 122 000. Polyacrylamide gel electrophoresis of the enzyme in the presence of sodium dodecyl sulfate suggests the enzyme is composed of subunits of molecular weights 44 000, 40 000, and 26 000 present in a molar ratio of 1:1:2. Incubation of the enzyme alone in the presence of [γ-^{32}P]ATP results in the phosphorylation of the 26 000-dalton subunit. Protein kinase II actively phosphorylates phosvitin, casein, and nonhistone chromosomal proteins but does not phosphorylate basic proteins such as histones or protamine to an appreciable extent. K_m values of 3.6 μM for ATP and 6.5 μM for GTP were determined in the presence of 4 mM Mg^{2+}. The enzyme is neither stimulated by cyclic adenosine 3′,5′-monophosphate or cyclic guanosine 3′,5′-monophosphate nor inhibited by the regulatory subunit of rabbit muscle protein kinase. Its activity is stimulated by KCl at concentrations below 0.2 M and inhibited by higher concentrations.

Considerable information is available concerning the structure and function of cAMP[1]-dependent protein kinases, whereas relatively little is known concerning those protein kinases not regulated by cyclic nucleotides (for review, see Rubin and Rosen, 1975). A number of cAMP-independent protein kinases have, however, been partially purified and characterized, including protein kinases from calf brain (Wålinder, 1973), rooster liver (Goldstein and Hasty, 1973), dogfish skeletal muscle (Blum et al., 1974), rat liver (Desjardins et al., 1972; Takeda et al., 1971; Ruddon and Anderson, 1972; Gamo and Lindell, 1974), human lymphocytes (Kemp et al., 1975), chick oviduct (Keller et al., 1976), and rat ascites cells (Dahmus, 1976). In addition a cAMP-independent protein kinase has been purified to homogeneity from baker's yeast (Lerch et al., 1975). The in vitro protein substrates of the cAMP-independent protein kinases are generally restricted to acidic proteins.

Of special interest is that class of protein kinases that function in the phosphorylation of nonhistone chromosomal (NHC) proteins. The association of specific NHC proteins with DNA appears to be a major factor in determining the selectivity of transcription in eukaryotic cells. This is supported by the observation that the transcriptional specificity of a variety of chromatin templates can be altered by transfer of the NHC proteins. Such reconstitution experiments have characterized the transcription of globin mRNA from mouse fetal and brain chromatin (Paul et al., 1973), of ovalbumin mRNA from oviduct chromatin from estrogen-stimulated and hormone-withdrawn chicks (Tsai et al., 1976), and of histone mRNA from HeLa cell chromatin isolated from cells at various stages in the cell cycle (Stein et al., 1975). In the latter case chromatin reconstituted with dephosphorylated NHC proteins from S phase cells was less active in the synthesis of histone mRNA than was chromatin reconstituted with untreated NHC proteins (Kleinsmith et al., 1976). These experiments perhaps provide the most direct evidence yet available that the state of phosphorylation of NHC proteins may be a key factor in the regulation of transcription. A variety of other observations including correlations of alterations of gene activity with changes in the phosphorylation of nuclear proteins (Kleinsmith et al., 1966; Ahmed, 1971; Jungmann and Schweppe, 1972; Allfrey et al., 1973; Johnson et al., 1974; Karn et al., 1974; Ahmed and Wilson, 1975; Kleinsmith, 1975), the stimulation of RNA synthesis in cell-free systems with phosphorylated NHC proteins (Teng et al., 1971; Shea and Kleinsmith, 1973; Kostraba et al., 1975), and the stimulation of RNA polymerase activity in cell-free systems with either partially purified or highly purified protein kinase (Jungmann et al., 1974; Martelo and Hirsch, 1974; Dahmus, 1976) further supports the idea that protein phosphorylation is intimately associated with gene regulation in eukaryotic cells.

An understanding of the role protein phosphorylation plays in the regulation of gene activity will require an understanding of the structure, function, and regulation of the protein kinases involved in the phosphorylation of NHC proteins. As a first step in this direction, we have purified and partially characterized the two major protein kinases present in Novikoff ascites tumor cells that phosphorylate acidic proteins. Whether or not these enzymes are indeed the protein kinases that phosphorylate NHC proteins in vivo has yet to be established.

The purification of the first of these enzymes, designated KI, has been reported previously (Dahmus, 1976). This protein kinase stimulates the activity of purified RNA polymerase II and does not appear to be regulated by cyclic nucleotides. In this communication we describe the purification and partial characterization of another major protein kinase active in the phosphorylation of acidic proteins.

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[1] Abbreviations used: cAMP and cGMP, cyclic adenosine and guanosine 3′,5′-monophosphate, respectively; NHC, nonhistone chromosomal; Tris, tris(hydroxymethyl)aminomethane; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; CM, carboxymethyl.

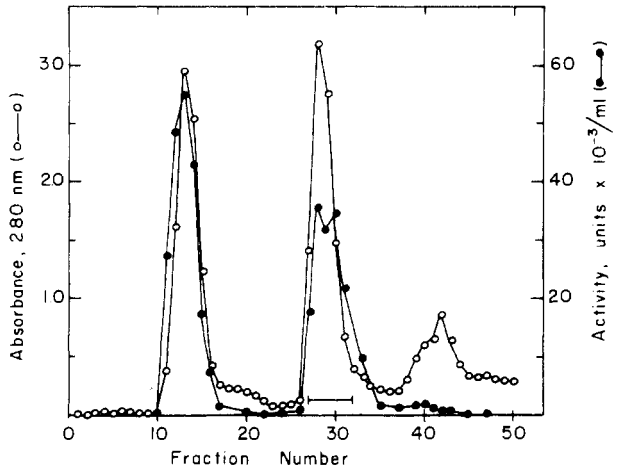

FIGURE 1: DEAE-cellulose chromatography of ascites tumor protein kinases I and II. The sample was applied to a 2.5 × 60 cm column of DEAE-cellulose equilibrated in buffer A containing 0.05 M $(NH_4)_2SO_4$. The column was developed with a step gradient of buffer A containing 0.05 M $(NH_4)_2SO_4$ (fractions 1-26), 0.18 M $(NH_4)_2SO_4$ (fractions 27-37), and 0.32 M $(NH_4)_2SO_4$ (fractions 38-50). Fractions of 10 mL were collected. Aliquots of 20 μL were assayed for protein kinase activity in the presence of 100 μg of casein. The bar indicates the fractions pooled.

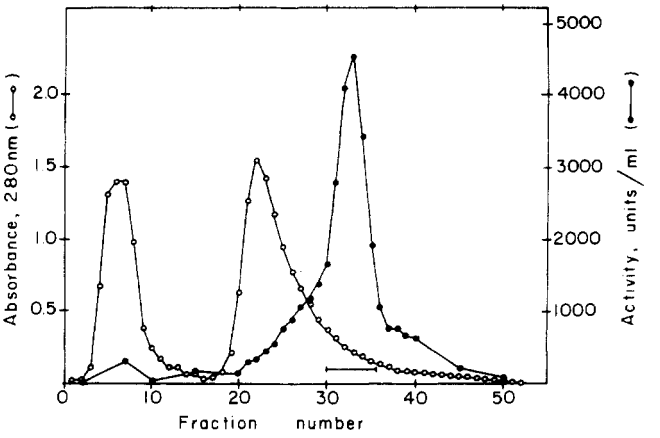

FIGURE 2: DEAE-Sephadex chromatography of ascites tumor protein kinase II. The enzyme was applied to a 1.5 × 30 cm column of DEAE-Sephadex and eluted with 3 column volumes of a linear gradient of 0.05-0.6 M $(NH_4)_2SO_4$ in buffer A. Fractions of 2 mL were collected.

Materials and Methods

Biochemicals. Unlabeled nucleoside triphosphates, CTP, GTP, UTP, and ATP, were obtained from P-L Biochemicals. [^{3}H]UTP was obtained from Schwarz/Mann. [γ-^{32}P]ATP was prepared by a modification (Walsh et al., 1971) of the procedure of Glynn and Chappell (1964). [γ-^{32}P]GTP was prepared by an adaptation of the procedure for ATP according to a suggestion by Dr. Earl Davies, University of Washington, Seattle.

Casein, obtained from Nutritional Biochemicals, was treated as described by Reimann et al. (1971). Phosvitin was obtained from Sigma. Bovine serum albumin was purchased from Schwarz/Mann. Protamine was obtained from Calbiochem. Histone 1 and histone 2b, prepared according to method 1 of Johns (1964), was a gift from Drs. W. Palmer and D. A. Walsh (Department of Biological Chemistry, University of California, Davis). Rabbit muscle phosphorylase *b*, purified according to the procedure of Fischer and Krebs (1962), was a gift from Dr. E. G. Krebs (Department of Biological Chemistry, University of California, Davis). Ascites tumor chromatin was prepared according to the procedure of Dahmus and McConnell (1969) and the NHC proteins were purified by a modification of the procedure of van den Brock et al. (1973). Chromatin was dissociated in the presence of 5 M urea, 3 M NaCl, 0.01 M Tris-HCl (pH 7.8), and 1 mM $PhCH_2SO_2F$, and the DNA was removed by centrifugation at 252 000*g* for 18 h. The heat-stable inhibitor protein, purified from rabbit skeletal muscle (Ashby and Walsh, 1974), was a gift from Mr. J. McPherson and Dr. D. A. Walsh. The regulatory and catalytic subunit of cAMP-dependent protein kinase, purified from rabbit skeletal muscle (Beavo et al., 1975), was a gift from Drs. P. J. Bechtel and E. G. Krebs.

Solutions. Buffer A contained 0.05 M Tris-HCl (pH 7.9), 25% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM dithiothreitol. Buffer B contained 0.05 M Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM dithiothreitol. Buffer C contained 0.05 M Tris-HCl (pH 7.9), 0.1 mM EDTA, and 0.5 mM dithiothreitol.

Cell Line. The Novikoff ascites tumor line was maintained by serial transplantation in female Sprague-Dawley rats. The cells were harvested and washed as previously described (Lee and Dahmus, 1973).

Protein Kinase Assay. The assay for protein kinase was carried out as previously described (Dahmus, 1976). One unit of activity is defined as the amount of enzyme that catalyzes the transfer of 1 pmol of phosphate from ATP to casein in 1 min under the standard assay conditions.

RNA Polymerase Assay. The assay for RNA polymerase activity was carried out as previously described (Dahmus, 1976).

Velocity Sedimentation. The sedimentation coefficient of ascites tumor protein kinase II was determined by sucrose gradient centrifugation as described by Martin and Ames (1961). Linear 5-20% sucrose gradients were prepared in buffer B containing 0.15 M KCl and centrifuged at 4 °C in the Beckman SW 40 rotor at 40 000 rpm for 16 h. In experiments involving ^{32}P-labeled enzyme, fractions were collected through the bottom of the tube and an aliquot of each fraction was precipitated with cold 10% trichloroacetic acid. An additional aliquot was assayed for protein kinase activity as described above. Bovine serum albumin ($s_{20,w}$ = 4.3) and *E. coli* alkaline phosphatase ($s_{20,w}$ = 6.3) were used as standards.

Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out in either 7.5 or 10% gels in sodium dodecyl sulfate according to the procedure of Weber and Osborn (1969). Gels were stained overnight in Coomassie blue, destained, and scanned in a Gilford recording spectrophotometer equipped with a linear transport. The molar ratio of subunits was determined from spectrophotometric tracings. Gels containing ^{32}P-labeled protein kinase were frozen, sliced into 1-mm sections, dried on small pieces of filter paper, and counted in a liquid scintillation spectrometer.

Cell Fractionation. Cytoplasmic and nuclear fractions were prepared by a modification of the procedure of Wu and Zubay (1974). Washed tumor cells were suspended in 5 volumes of 120 mM KCl-5 mM magnesium acetate-7 mM β-mercaptoethanol-30 mM Tris-HCl (pH 7.5), allowed to sit for 10-15 min, and then centrifuged at 4300*g* for 10 min. The pellet was suspended and allowed to swell for 15 min in 2 volumes of 5 mM KCl-0.75 mM magnesium acetate-5 mM Tris-HCl (pH 7.5). The suspension was then homogenized in small volumes (12-14 mL) by two strokes of a Dounce homogenizer (pestle size A, tight fitting) to rupture the outer cell membrane. Following the addition of 0.1 volume of 1.27 M KCl-48 mM magnesium acetate-20 mM β-mercaptoethanol-280 mM

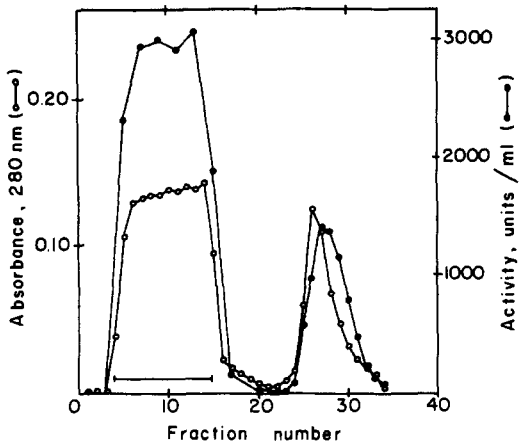

FIGURE 3: CM-cellulose chromatography of ascites tumor protein kinase II. The enzyme was applied to a 1.5 × 12 cm column of CM-cellulose equilibrated in buffer B. The column was developed with 3 column volumes of a linear gradient of 0–0.6 M NH_4Cl in buffer B. Fractions of 2 mL were collected.

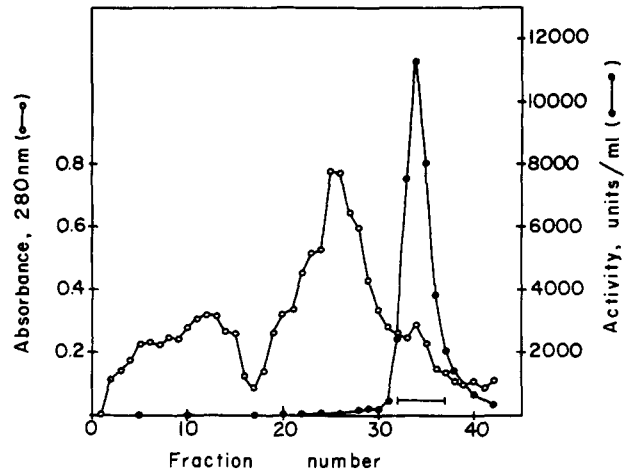

FIGURE 4: Phosphocellulose chromatography of ascites tumor protein kinase II. The enzyme was applied to a 0.9 × 10 cm column of phosphocellulose and eluted with 5 column volumes of a linear gradient of 0.05–1.2 M NH_4Cl in buffer C. Fractions of 1 mL were collected.

Tris-HCl (pH 7.5), the sample was centrifuged at 4300*g* for 15 min. The nuclear pellet was suspended in an equal volume of 120 mM KCl–5 mM magnesium acetate–7 mM β-mercaptoethanol–30 mM Tris-HCl (pH 7.5) and centrifuged at 4300*g* for 15 min. The supernatant of this centrifugation was combined with the previous supernatant to constitute the cytoplasmic fraction. The washed nuclear pellet was suspended in 3 volumes of 2.4 M sucrose–25 mM KCl–5 mM $MgCl_2$–0.5 mM dithiothreitol–0.01 M Tris-HCl (pH 7.9) and centrifuged at 35 000 *g* for 1 h in the Sorvall SS-34 rotor. The nuclear layer was then suspended in an equal volume of 0.02 M Tris-HCl (pH 7.9)–2.0 M sucrose–10 mM $MgCl_2$–1 mM dithiothreitol.

Aliquots of the cytoplasmic and nuclear fractions were assayed for alkaline phosphatase (Weiser, 1973), glyceraldehyde-3-phosphate dehydrogenase (Bruening et al., 1970), and cytochrome *c* reductase (Fleischer and Fleischer, 1967). DNA content was determined by the procedure of Burton (1968) following the precipitation of total nucleic acid with cold 10% trichloroacetic acid and removal of RNA according to Schmidt and Taunhauser (1945).

Protein Determination. Protein concentrations were determined by the method of Lowry et al. (1951) after precipitation of aliquots with cold 10% trichloroacetic acid.

Results

Purification of Novikoff Ascites Tumor Protein Kinase II. All steps were carried out at 4 °C. The early steps of the purification are an adaptation of the procedure originally described by Roeder and Rutter (1970) for the purification of eukaryotic RNA polymerases. This procedure allows the recovery of ascites tumor RNA polymerases I and II as well as two major cAMP-independent protein kinases active in the phosphorylation of acidic proteins. Early steps in the purification were carried out in the presence of buffer containing 25% glycerol. RNA polymerase is stabilized by the presence of glycerol and it need be present only up to the stage of resolution of protein kinase and polymerase activity.

Fifty grams of Novikoff ascites tumor cells was routinely used for each preparation. The cells were suspended in an equal volume of 0.02 M Tris-HCl (pH 7.9)–2.0 M sucrose–10 mM $MgCl_2$–1 mM dithiothreitol with the aid of a Teflon homogenizer. The solution was then brought to 0.3 M $(NH_4)_2SO_4$ by the addition of 4 M $(NH_4)_2SO_4$ adjusted to pH 7.9, and the cells were allowed to lyse for 10 min with occasional stirring. The lysed cells were then sonicated with 10-s bursts at 160 W in a Bronwill Biosonic IV sonicator, until the solution dropped freely from the tip of a Pasteur pipette. The suspension was then diluted with 2 volumes of buffer A and centrifuged at 142 800*g* for 3 h. The supernatant was filtered through sintered glass and precipitated with 30% saturated $(NH_4)_2SO_4$. The precipitate was discarded and the $(NH_4)_2SO_4$ concentration of the supernatant increased to 50% saturation. After at least 3 h at 0 °C the precipitate was recovered by centrifugation at 31 000*g* for 30 min and dissolved in buffer A. The solution was dialyzed overnight against buffer A containing 0.05 M $(NH_4)_2SO_4$ and clarified by centrifugation at 27 000*g* for 15 min.

DEAE-Cellulose Chromatography. The solution was passed through a column of DEAE-cellulose equilibrated with buffer A containing 0.05 M $(NH_4)_2SO_4$. About 60% of the protein kinase activity (KI) does not bind to the resin and is recovered in the flow through peak. The remaining protein kinase activity (KII) as well as RNA polymerases I and III were eluted with buffer A containing 0.18 M $(NH_4)_2SO_4$ (Figure 1). RNA polymerase II was eluted with buffer A containing 0.32 M $(NH_4)_2SO_4$. Fractions containing protein kinase II activity were pooled and dialyzed against buffer A containing 0.05 M $(NH_4)_2SO_4$.

DEAE-Sephadex Chromatography. To resolve KII from RNA polymerase I, the sample was chromatographed on DEAE-Sephadex as described in Figure 2. The major fraction of protein kinase eluted after the main protein peak at about 0.25 M $(NH_4)_2SO_4$ and was completely resolved from RNA polymerase I which eluted at about 0.1 M $(NH_4)_2SO_4$. The peak of protein kinase activity was dialyzed against buffer B.

CM-Cellulose Chromatography. To remove an additional protein kinase present in this fraction, the sample was chromatographed on CM-cellulose as described in Figure 3. The major peak of protein kinase, designated KII, appeared in the flow through peak. An additional protein kinase that did bind to the column was eluted with a gradient of NH_4Cl. Fractions containing KII were pooled and applied directly to phosphocellulose.

Phosphocellulose Chromatography. The sample was chromatographed on phosphocellulose as described in Figure 4. Protein kinase activity emerged as a single peak at 0.8 M

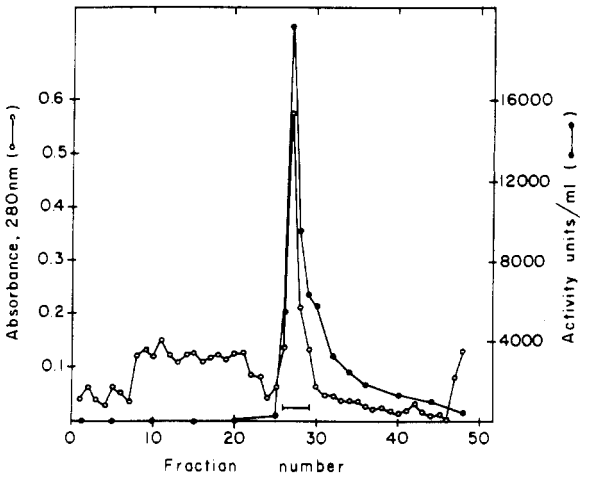

FIGURE 5: Hydroxylapatite chromatography of ascites tumor protein kinase II. The enzyme was applied to a 0.9 × 5 cm column of hydroxylapatite and eluted with seven column volumes of a linear gradient of 0.01–0.8 M potassium phosphate, pH 7.2, containing 0.5 mM dithiothreitol. Fractions of 1 mL were collected.

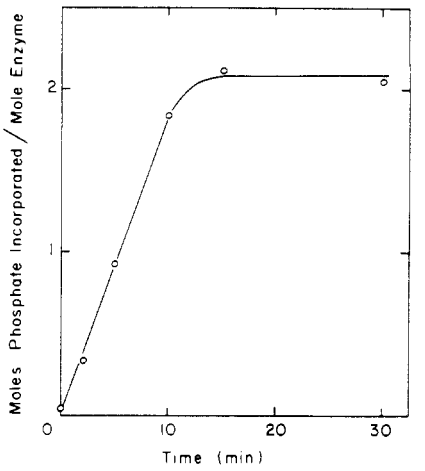

FIGURE 6: Incorporation of ^{32}P by ascites tumor protein kinase II in the absence of added protein substrate. The standard reaction mixture contained 10 μg of KII and 0.125 mM [γ-^{32}P]ATP (specific activity, 807 cpm/pmol) in a final volume of 0.32 mL. At the indicated time, 0.05-mL aliquots were removed and incorporation was determined as described in Materials and Methods.

TABLE I: Summary of Purification of Novikoff Ascites Tumor Protein Kinase II.[a]

Step	Vol (mL)	Protein (mg)	Units	Spec Act.
1. Cell lysate	98	637	64 100	101
2. 230 000*g* supernatant	90	454	95 000	209
3. 30–50% saturated $(NH_4)_2SO_4$	24	154	46 700	303
4. DEAE-cellulose	50	100	82 600	826
5. DEAE-Sephadex	29	4.99	65 800	13 200
6. CM-cellulose	30	3.24	70 200	21 700
7. Phosphocellulose	4.8	0.157	38 200	243 000
8. Hydroxylapatite	4.3	0.069	36 000	517 000

[a] This preparation utilized 15 mL of packed cells. Reaction conditions are as described in Materials and Methods

NH_4Cl. Peak fractions were pooled and applied, without dialysis, to hydroxylapatite.

Hydroxylapatite Chromatography. Enzyme was chromatographed on hydroxylapatite as described in Figure 5. A single peak of protein kinase emerged at about 0.15 M potassium phosphate. Peak fractions were pooled and dialyzed against buffer B.

Table I summarizes the results obtained from a typical purification.

Molecular Weight and Subunit Composition. When purified enzyme was centrifuged in a linear 5–20% sucrose gradient, the protein kinase activity sedimented as a 7.6S component and was coincident with the protein peak. Chromatography of the purified enzyme on Bio-Gel A-1.5m also resulted in a single coincident peak of protein and protein kinase activity. A Stokes radius of 39 Å was determined. A molecular weight of 122 000 was calculated from the sedimentation and gel filtration data (Siegel and Monty, 1966). The constant specific activity across the peak following chromatography on hydroxylapatite or Bio-Gel A-1.5m, as well as sucrose gradient centrifugation, suggests that the major fraction of protein is indeed protein kinase. A variety of buffer systems has been used for nondenaturing gel electrophoresis but a satisfactory system has not been developed. At pH 8.3 the enzyme fails to penetrate the gel, whereas under acid conditions it migrates with the dye front.

Polyacrylamide gel electrophoresis of KII in the presence of sodium dodecyl sulfate results in the resolution of three protein bands. The molecular weights of the proteins, determined by mobility relative to standards of known molecular weight, are 44 000, 40 000, and 26 000. Since no protein was determined in the region of the gel corresponding to a molecular weight of 122 000, these proteins appear to be enzyme subunits. The molar ratio of subunits, determined from gel scans of Coomassie blue stained gels, is 1:1.11 ± 0.06:1.86 ± 0.11. If we, therefore, assume the molar ratio of subunits to be 1:1:2, the molecular weight of the holoenzyme is 136 000. This is in agreement with the molecular weight of 122 000 determined by sedimentation velocity and gel filtration.

Phosphorylation of KII. Purified KII catalyzes the incorporation of γ-^{32}P from ATP into protein in the absence of added protein substrate. The kinetics of this incorporation are shown in Figure 6. Assuming a molecular weight of 136 000, about 2 mol of phosphate is incorporated per mol of enzyme. An aliquot of this reaction mixture was applied to a 5–20% sucrose gradient and centrifuged as described in the legend to Figure 7. The gradient was then assayed for both protein kinase activity and the position of labeled phosphate. It is clear that the ^{32}P incorporated by enzyme alone sediments with the protein kinase activity. The endogenous activity is therefore likely due to the phosphorylation of the enzyme itself rather than a contaminating protein. Polyacrylamide gel electrophoresis of the ^{32}P-labeled KII in the presence of sodium dodecyl sulfate shows that the 26 000-dalton subunit served as phosphate acceptor (Figure 8). The fact that the 26 000-dalton subunit is phosphorylated and that 2 mol of phosphate is incorporated per mol of enzyme is consistent with the observation that the holoenzyme contains two 26 000-dalton subunits.

Substrate Specificity of KII. The ability of protein kinase II to phosphorylate a variety of proteins was determined (Table II). Of the proteins tested, phosvitin was the preferred substrate, although casein and NHC proteins were also actively phosphorylated. Basic proteins such as histones and protamine were not phosphorylated to an appreciable extent.

Experiments were also performed to examine nucleotide substrate specificity. Initial reaction rates were determined in

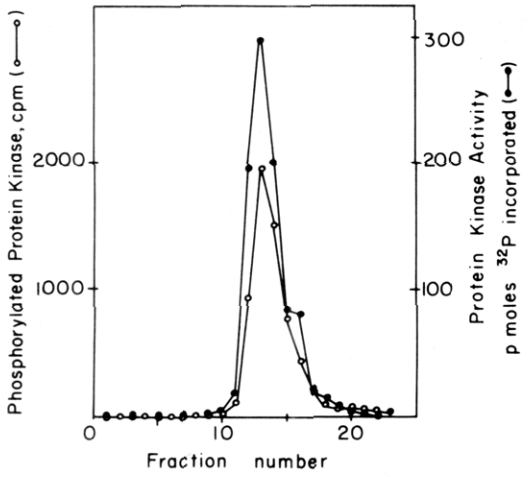

FIGURE 7: Sucrose velocity sedimentation of ^{32}P-labeled ascites tumor protein kinase II. KII was phosphorylated as described in the legend to Figure 6. A sample containing 3 μg of ^{32}P-labeled KII was layered on a 13-mL 5–20% sucrose gradient in buffer B containing 0.15 M KCl and centrifuged for 16 h at 40 000 rpm at 4 °C in a Beckman SW40 rotor. Fractions of 0.5 mL were collected from the bottom. Aliquots of 0.04 mL were precipitated with trichloroacetic acid to determine the position of ^{32}P-labeled protein. Aliquots of the same size were assayed for protein kinase activity.

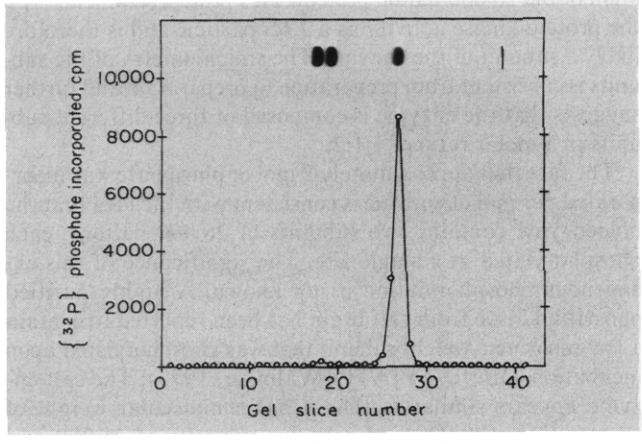

FIGURE 8: Sodium dodecyl sulfate gel electrophoresis of ^{32}P-labeled ascites tumor protein kinase II. KII was phosphorylated as described in the legend to Figure 6 and electrophoresed on 7.5% sodium dodecyl sulfate gels as described in Materials and Methods. The gel was sliced and counted in a liquid scintillation spectrometer.

the presence of various concentrations of either [γ-^{32}P]ATP or [γ-^{32}P]GTP and the data analyzed according to Lineweaver and Burk (1934). In the presence of 4 mM Mg^{2+}, with casein as phosphate acceptor, the apparent K_m for ATP was 3.6 μM and 6.5 μM for GTP. The V_{max} determined in the presence of GTP was identical with that determined in the presence of ATP.

Effect of cAMP and Regulatory Proteins on Protein Kinase II. No change in activity was observed when protein kinase II was assayed in the presence of cAMP or cGMP over a concentration range of 10^{-6} to 10^{-4} M. We also examined its sensitivity to the heat-stable inhibitor of cAMP-dependent protein kinases (Ashby and Walsh, 1974) and the regulatory subunit of rabbit muscle protein kinase (Beavo et al., 1975). At comparable enzymatic activity, no inhibition of ascites tumor protein kinase II was observed at heat-stable inhibitor concentrations which resulted in greater than 95% inhibition of the free catalytic subunit of rabbit muscle protein kinase.

TABLE II: Phosphorylation of Various Protein Substrates by Protein Kinase II.[a]

Protein Substrate	Protein Kinase Act. (pmol ^{32}P incorp per 10 min)	Rel Act. (%)
Phosvitin	905	100
Casein	323	35.9
Nonhistone chromosomal proteins	74.5	8.2
Histone 1	17.9	2.0
Histone 2b	10.9	1.2
Protamine	2.9	0.32
Bovine serum albumin	1.0	0.11
Egg white lysozyme	1.1	0.13
Phosphorylase *b*	1.1	0.12

[a] The assays were performed under standard assay conditions in the presence of 100 μg of protein substrate. Background values of less than 1 pmol were subtracted for each substrate except nonhistone chromosomal proteins (2.2 pmol), histone 1 (3.7 pmol), histone 2b (4.3 pmol), and protamine (1.3 pmol).

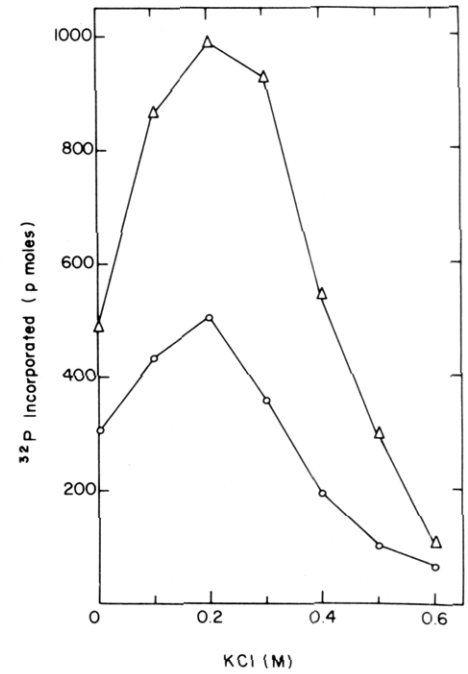

FIGURE 9: Effect of KCl on the activity of ascites tumor protein kinase II. Increasing concentrations of KCl were added to the standard assay mixtures before initiation of the reaction with protein kinase. Activity with casein serving as protein substrate (○—○), with phosvitin as protein substrate (△—△).

Furthermore, no inhibition of ascites tumor protein kinase II was observed at concentrations of regulatory subunit sufficient to inhibit the homologous catalytic subunit by greater than 95%.

Effect of Increasing Salt Concentration on Protein Kinase. The activity of protein kinase II was markedly influenced by the presence of salt. The effect of KCl on protein kinase II activity in the presence of phosvitin or casein is shown in Figure 9. The activity is stimulated by low concentrations of KCl with an optimum of about 0.2 M. Higher KCl concentrations result in a rapid decline in activity. The addition of $MnCl_2$ resulted in inhibition of protein kinase activity. Fifty percent inhibition of activity was obtained at about 0.3 mM $MnCl_2$ in the presence of 4 mM magnesium acetate and 0.125 mM ATP. Inhi-

TABLE III: Subcellular Distribution of Protein Kinase II.[a]

	Percent Activity	
	Cytoplasmic	Nuclear
Protein kinase II	76	24
DNA[b]	11	89
Protein[b]	90	10
RNA polymerase I	86	14
RNA polymerase II	20	80
Alkaline phosphatase	82	18
Glyceraldehyde-3-phosphate dehydrogenase	99	1
Cytochrome *c* reductase	86	14

[a] Protein kinase II was purified through CM-cellulose chromatography, from both the cytoplasm and nuclear fractions, as described in Materials and Methods. The proportion of RNA polymerases I and II was estimated from the relative sizes of the peaks observed following DEAE-cellulose chromatography. Even though RNA polymerases I and III are not resolved by DEAE-cellulose, polymerase III constitutes less than 5% of the enzyme present in this fraction. Other assays were performed, as described in Materials and Methods, on the cytoplasmic and nuclear fractions without further purification. [b] Percent distribution is based on total mass.

bition beyond 80% could not be obtained at $MnCl_2$ concentrations as high as 4 mM.

Subcellular Distribution of Protein Kinase II. Ascites tumor cells were homogenized and the subcellular fractions isolated by differential centrifugation as described in Materials and Methods. The distribution of DNA, alkaline phosphatase, glyceraldehyde-3-phosphate dehydrogenase, and cytochrome *c* reductase indicates a relatively clean resolution of the cytoplasmic and nuclear fraction (Table III). The activity of protein kinase II was determined following purification of the enzyme through CM-cellulose. Since no additional protein kinase is resolved following CM-cellulose chromatography, this should give an accurate reflection of the amount of KII present in the nuclear and cytoplasmic fractions.

Although the major proportion of protein kinase II activity appeared in the cytoplasmic fraction, a significant proportion, as great as 32% in some experiments, was found associated with the nuclear fraction. The total yield of enzyme was comparable to that obtained starting with whole cells. From the distribution of RNA polymerase I, it appears that loss of nuclear enzymes into the cytoplasm, through leaching, must have taken place since localization of the DNA nearly exclusively in the nucleus indicates that contamination by nuclear breakage is minimal. It has been reported that $PhCH_2SO_2F$ inhibits the release of RNA polymerase I from isolated rat liver nuclei (Lindell, 1975). We found no significant change in the distribution of protein kinase II or RNA polymerase when the purification was done in the presence of 2 mM $PhCH_2SO_2F$. Even though it is not possible to make a definitive statement concerning the localization of KII, it is likely that a significant proportion is contained within the nucleus.

Effect of Protein Kinase II on the Activity of RNA Polymerases I, II, and III. We have previously reported that RNA polymerase II and, to a lesser extent, RNA polymerase I are stimulated by protein kinase I (Dahmus, 1976). We, therefore, examined the effect of purified KII on the activities of RNA polymerases I, II, and III. RNA polymerases was purified from Novikoff ascites tumor cells as previously described (Dahmus, 1976). We have been unable to demonstrate an effect on the rate of RNA synthesis catalyzed by RNA polymerases I, II, or III by various concentrations of KII. We have not determined the extent of polymerase phosphorylation prior to assay and therefore cannot eliminate the possibility that RNA polymerase can serve as a substrate for KII.

Discussion

The procedure developed for the purification of protein kinase II also allows for the purification of RNA polymerases I and II and protein kinase I. Upon chromatography on DEAE-cellulose, protein kinase I appears in the flow through peak, protein kinase II and RNA polymerases I and III are eluted as a single peak, and RNA polymerase II is eluted at a higher salt concentration. RNA polymerase I is resolved from protein kinase II and RNA polymerase III by chromatography on DEAE-Sephadex.

Novikoff ascites tumor protein kinase II, after a greater than 5000-fold purification, has a specific activity of about 0.5 μmol of phosphate transferred per min per mg of enzyme. Protein kinase I, purified from these cells, has a specific activity of about 1 μmol of phosphate transferred per min per mg of enzyme (Dahmus, 1976).

We have not found a buffer system suitable for electrophoresis of KII under nondenaturing conditions. For this reason, the assignment of subunit composition remains tentative. Incubation of purified KII in the presence of ATP but in the absence of additional protein results in the phosphorylation of the 26 000-dalton protein. This protein sediments with the protein kinase activity as a 7.6S particle and is therefore likely a subunit of the enzyme. The stoichiometry of the subunits is consistent from preparation to preparation and further suggests that the enzyme is composed of three different subunits in a molar ratio of 1:1:2.

The fact that approximately 2 mol of phosphate was incorporated per mol of enzyme is consistent with the idea that the holoenzyme contains two subunits of 26 000 daltons, each phosphorylated at a single site. The significance of this endogenous phosphorylation is not known. A highly purified phosvitin kinase from calf brain has been reported to contain a low-molecular-weight subunit that was phosphorylated upon incubation with [γ-^{32}P]ATP (Wålinder, 1973). The calf enzyme appears similar in that it has a molecular weight of greater than 100 000 and contains subunits of 41 000 and 26 000 daltons. It is also of interest that purified beef heart and brain cAMP-dependent protein kinase catalyze the incorporation of phosphate from ATP into its regulatory subunit (Erlichman et al., 1974; Maeno et al., 1974).

Rabbit skeletal muscle phosphorylase kinase exists in phosphorylated and nonphosphorylated forms that differ in their catalytic properties (DeLange et al., 1968). Conversion of the inactive nonphosphorylated form to the activated form can be brought about by either self-phosphorylation in the presence of ATP and magnesium ions, or by phosphorylation catalyzed by a cAMP-dependent protein kinase (Walsh et al., 1971). The autocatalytic component of the activation reaction is stimulated by calcium ions. It is also of interest that rabbit muscle phosphorylase kinase is composed of three types of subunits (Hayakawa et al., 1973). Although the molecular weight of the holoenzyme and subunits are clearly different from that of ascites tumor protein kinase II, the overall structural similarity raises the possibility that KII may be subject to similar types of regulation.

Ascites tumor protein kinase II catalyzes the phosphorylation of acidic proteins such as casein, phosvitin, or NHC proteins but does not phosphorylate basic proteins such as histones or protamine to an appreciable extent. Either ATP or GTP can be utilized as a phosphate donor. The ability of KII

to utilize GTP allows it to easily be distinguished from KI (Dahmus, 1976). The stimulation of KII activity by low salt is characteristic of a number of protein kinases that phosphorylate acidic proteins (Desjardins et al., 1972; Traugh and Traut, 1974; Rodnight and Lavin, 1964; Kemp et al., 1975).

Ascites tumor protein kinase II has properties similar to rat liver nuclear casein kinase NII reported by Desjardins et al. (1972). Both enzymes are insensitive to cAMP, are stimulated by KCl at low concentrations, and have similar chromatographic properties on phosphocellulose and DEAE-substituted resins. KII also resembles protein kinase CKI from human lymphocytes (Kemp et al., 1975), kinase IIIc from rabbit reticulocytes (Traugh and Traut, 1974), and phosvitin kinase from ox brain (Rodnight and Lavin, 1964). A protein kinase of similar properties has also been shown to be associated with highly purified rat liver nucleoli (Gamo and Lindell, 1974).

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Purification and Characterization of a Plasminogen Activator Secreted by Cultured Human Pancreatic Carcinoma Cells†

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ABSTRACT: A plasminogen activator secreted by cultured human pancreatic carcinoma (Mia PaCa-2) cells has been purified to apparent homogeneity by procedures including Sepharose-L-arginine methyl ester affinity chromatography, Sephadex G-200 gel filtration, isoelectric focusing, and sodium dodecyl sulfate gel electrophoresis. The plasminogen activator shares many properties with urokinase including: molecular weight (55 000), isoelectric point (8.7), heat stability (60 °C, 30 min), pH stability (1.5-10), and its mode of activation of plasminogen. The intracellular enzyme is membrane bound and can be solubilized by detergent. Solubilized activator has a molecular weight similar to that of the secreted enzyme as determined by sodium dodecyl sulfate gel electrophoresis. The production of plasminogen activator by Mia PaCa-2 cells is totally inhibited by actinomycin D and cycloheximide.

Many publications appeared recently describing increased fibrinolytic activity associated with viral transformation. Unkeless et al. (1973) and Ossowski et al. (1973) described increased fibrinolytic activity in chick embryo fibroblast after transformation by Rous sarcoma virus and in hamster fibroblast after transformation by SV-40 virus. In subsequent studies, they provided evidence that their fibrinolytic system consisted of two proteins: a serum factor from the medium identified as plasminogen (Quigley et al., 1974), and a cell factor, a specific serine protease which functioned as a plasminogen activator (Unkeless et al., 1974). The activator secreted by SV-40 transformed hamster fibroblast has also been purified and characterized by Christman and Acs (1974). Recently, Chen and Buchanan (1975) reported on a plasminogen-independent fibrinolytic activity secreted by Rous sarcoma transformed chicken fibroblast cells.

In an earlier communication Yunis et al. (1973) reported the secretion of "fibrinolysin" by rat ovarian tumor cells in culture. In subsequent studies, we identified in conditioned serum-free medium, prepared from cultured rat breast carcinoma R2426 cells, two separable fibrinolytic activities, a direct fibrinolysin and a plasminogen activator (Wu et al., 1975; Schultz et al., 1975). The direct fibrinolysin is distinct from plasmin both biochemically and immunologically. The activator is similar to urokinase in molecular size, mode of activation of plasminogen, and other properties (Wu et al., 1976).

This report will describe the purification and characterization of a plasminogen activator from conditioned serum-free medium of cultured human pancreatic carcinoma cells.

Materials and Methods

Human plasminogen was prepared from fresh plasma by the procedure of Deutsch and Mertz (1970). Urokinase, Trasylol, and streptokinase were purchased from Calbiochem, La Jolla, Calif.; thrombin was from Upjohn, Kalamazoo, Mich.; human fibrinogen was from Cutter, Berkeley, Calif.; *p*-tosyl-*N*-arginine methyl ester, Sepharose 4B, arginine methyl ester, *N*-α-aceryl-L-lysine methyl ester, soybean trypsin inhibitor, Sephadex, and Triton X-100 were from Sigma Chemical Co., St. Louis, Mo.; cyanogen bromide was from Eastman, Rochester, N.Y.; Ampholine was from LKB, Rockville, Md.; acrylamide, bisacrylamide, and sodium dodecyl sulfate were from Bio-Rad, Richmond, Calif. Culture media, fetal calf serum, and horse serum were purchased from GIBCO, Grand Island, N.Y. Dulbecco's modified Eagle medium (DME)[1] was prepared from powder form and fortified with 10% fetal calf serum and 2.5% horse serum (DME-HSFC).

Cell Culture. Human pancreatic carcinoma cell line, Mia PaCa-2 (Yunis et al., 1977), was established according to previously described procedures (Wu et al., 1975; Yunis et al., to be published). Cells are grown in Dulbecco's modified Eagle's medium fortified with 10% fetal calf serum and 2.5% horse serum.

Preparation of Serum-Free Conditioned Medium. Serum-free conditioned medium from cultured pancreatic carcinoma was prepared as previously described (Wu et al., 1975). When cell growth had reached confluence, the medium was aspirated and the plates were rinsed three times with

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[1] Abbreviations used: DME, Dulbecco's modified Eagle medium; HSFC, 2.5% horse serum-10% fetal calf serum; SFCM, serum-free conditioned medium; Tame, *N*-*p*-tosyl-L-arginine methyl ester; Tris, tris(hydroxymethyl)aminomethane; Alme, *N*-α-acetyl-L-lysine methyl ester; EDTA, ethylenediaminetetraacetic acid.