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PROPERTIES OF RAT LIVER NUCLEAR PROTEIN KINASES

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Summary

Two cyclic nucleotide-independent protein kinases (ATP:protein phosphotransferase, EC 2.7.1.37) have been purified to homogeneity from rat liver nuclei. While these enzymes have many similar catalytic properties (preference for acidic rather than basic proteins), they differ in molecular weight and subunit composition. Protein kinase NII will utilize ATP and GTP as phosphate donors while protein kinase NI will only effectively use ATP. Both enzymes reveal an unusual activation by Fe^{2+}.

Introduction

Considerable interest in acidic nuclear protein phosphorylaction has been evident in the past few years because many of these proteins are phosphorylated under conditions which suggest a potential role in the regulation of gene transcription [1—8]. In an effort to characterize the enzymes participating in this process, numerous reports have appeared describing the enzymic properties of protein kinases (ATP:protein phosphotransferase, EC 2.7.1.37) found in rat liver nuclei [9—15] and from other sources. Most studies report that these protein kinases are not activated by cyclic nucleotides. Most of these previous studies have been performed with partially purified preparations; however, recently three cyclic nucleotide-independent enzymes have been purified to homogeneity [16—18]. In two of these studies, however, whole cells rather

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Abbreviation: EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

than nuclei were employed as the source of the initial extraction.

This laboratory has recently reported the purification to homogeneity of two of these protein kinases from rat liver nuclei [21,22]. Evidence is presented in this paper which suggests that although they are very similar in many catalytic properties, proteins kinases NI and NII both have unique subunit structures indicating that these are two distinct enzymes. While it is known that both acidic and basic proteins are phosphorylated in the nucleus, it is suggested that one (or both) of these enzymes are likely to play a major role in the phosphorylation of non-histone proteins of rat liver nuclei.

Methods

Chemicals and materials. Chemicals were purchased from the following sources: hydrolyzed and partially dephosphorylated casein, α-casein, phosvitin, histones, protamine sulfate, beef heart cyclic AMP-dependent protein kinase, Sigma; ATP, GTP, PL-Biochemicals; [γ-^{32}P]ATP and [γ-^{32}P]GTP (10 Ci/mmol), New England Nuclear. All other chemicals were purchased in the highest grade possible. Cellulose phosphate paper (P-81 chromatography) and 3 MM filter paper (2.3 cm) were purchased from Whatman. The synthetic heptapeptide kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was obtained from Vega-Fox Biochemicals, Tucson, AZ. Heat-stable inhibitory preparations were isolated from rat liver by the method of Walsh et al. [23].

Protein kinase assay. Variations of the protein kinase assay originally described by Desjardins et al. [9] were used. Reactions were started by the addition of 50 μl enzyme which was in 0.05 M Tris-HCl buffer (pH 7.9), 5.0 mM $MgCl_2$, 1.0 mM EDTA, 1.0 mM dithiothreitol and 10% glycerol. Final assays (0.1 ml) for protein kinase NI activity contained: 0.1 mM ATP or GTP, 0.2 M NaCl, 0.05 M Tris (pH 7.2), 5.0 mM $MgCl_2$, 0.2 mM EDTA, 0.3 mM EGTA, α-casein (12 mg/ml) and 0.1 μCi of labeled nucleoside triphosphate. Assays for protein kinase NII activity were identical except they contained phosvitin (2 mg/ml) as substrate.

Protein kinase assays were incubated at 30°C for 30 min. Reactions were terminated by two different procedures depending on the substrate employed. 80-μl aliquots were spotted onto: (1) Whatman 3 MM filters (2.3 cm) which were then processed as previously described [9], or (2) cellulose phosphate strips (2 × 2 cm) according to the procedure of Witt and Roskoski [24]. For assays with histone, the cellulose phosphate papers were washed in water (50 ml/sample), once for 10 min, three times for 5 min with water followed by acetone and ether (2 ml sample). For assays with the synthetic peptide kemptide, the cellulose phosphate paper were washed once for 10 min and three times for 5 min with 30% (v/v) acetic acid (25 ml/sample) followed by acetone and ether (2 ml/sample).

Results

Studies described in this paper were performed on enzyme which had been previously prepared and stored in liquid nitrogen. Although both enzymes retain activity after freezing and thawing, the effects of repeated freezing and

thawing and long-term freezing are not known. These studies were therefore performed using enzymes which had been frozen for only a few weeks.

Subunit composition

Determinations of the subunit composition of protein kinases NI and NII had previously revealed an apparent similarity of the molecular weights of the β subunits of protein kinase NII with those of protein kinase NI. Fig. 1 shows that when a mixture of the two enzymes is subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, the low molecular weight subunits of protein kinase NII are clearly resolved from those of protein kinase NI. With the 10% polyacrylamide gel system employed here, the molecular weight of protein kinase NI is 28 000 while the β subunits of protein kinase NII are 23 000 which is slightly lower than the previously reported estimate obtained with 7.5% polyacrylamide gels [21]. The α and α', subunits of protein kinase NII are 42 000 and 39 000 daltons, respectively [21].

Substrate specificities

As previously reported, protein kinases NI and NII exhibit maximal activity using casein and phosvitin substrates respectively. Both enzymes prefer these

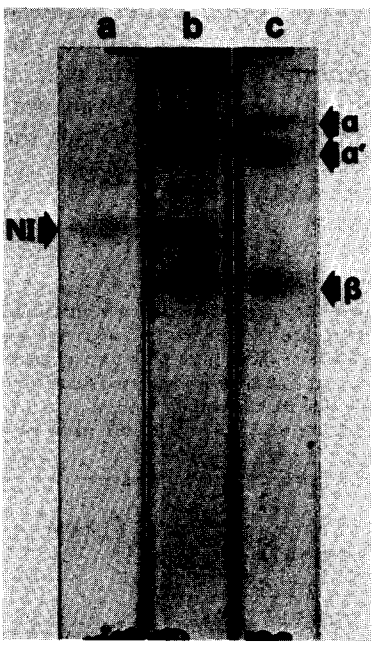

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of nuclear protein kinases. Aliquots of purified protein kinases NI and NII were removed from liquid nitrogen, thawed, and precipitated by the addition of cold 10% trichloroacetic acid. Protein standards were also treated in the same way. Standard proteins and their molecular weights were: serum albumin (dimer and monomer), ovalbumin, chymotrypsinogen, and myoglobin with respective molecular weights of: 136 000, 68 000, 43 000, 25 700 and 17 200. The precipitates were collected by centrifugation (Brinkman Microfuge), redissolved in sample buffer, and applied to 0.5 × 7 cm gels consisting of 10% polyacrylamide. They were subjected to electrophoresis according to the procedure of Weber and Osborne [25]. The amount of material analyzed represents (a) half of a preparation of protein kinase NI (approximately 1 μg; (c) 1/30th of a preparation of protein kinase NII (4.5 μg) or (b) a mixture of the same sized aliquots of both enzymes.

acidic protein substrates over basic histone substrates. To better understand the types of proteins phosphorylated by these enzymes, a wide range of substrates was examined. It can be seen in Table I that protein kinases NI and NII differ in substrate preference in addition of the already observed preference for casein and phosvitin. The order of substrate preference exhibited by protein kinase NI is: casein > phosvitin > H1 > H2b > mixed histone > H3, H2a, protamine; while that for protein kinase NII is: phosvitin > casein > H1 > H2b, H2a, H3, mixed histone, protamine. The order of substrate preference exhibited by a commercial preparation of cyclic AMP-dependent protein kinase from bovine heart was also determined for comparison with the two cyclic nucleotide-independent rat liver nuclear protein kinases. The order of substrate preference for this enzyme was: H1 > mixed histone > H2a > protamine > H3 > H2b, casein phosvitin.

When assays are conducted employing the cellulose phosphate papers (P-81) rather than 3 MM filters, the substrate preferences of all three enzymes for individual histones and protamine are somewhat altered. Most notably, the assays with ion-exchange paper reveals a more substantial utilization of protamine and histones H1 and H2b by protein kinase NII and cyclic AMP-dependent enzyme. The synthetic heptapeptide kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) is a useful model substrate for the cytoplasmic cyclic AMP—dependent protein kinase [26]. This synthetic peptide becomes the preferred substrate of the cyclic AMP-dependent enzyme under our assay conditions. This substrate is phosphorylated at a low level by protein kinase NI; however, it is ineffective as a substrate for protein kinase NII. It can also be seen that protein kinase NII utilizes GTP as well as ATP in its reactions, and that the

TABLE I

SUBSTRATE SPECIFICITIES OF NUCLEAR AND CYTOPLASMIC PROTEIN KINASES

All substrates were present at 1 mg/ml and each set of assays was corrected for background or endogenous phosphorylation in the absence of substrate. Relative activities of protein kinases NI and NII are expressed as a percentage of control obtained using the preferred substrate α-casein or phosvitin, respectively, when assayed using 3MM filters. The cyclic AMP-dependent enzyme activity is expressed relative to the substrate H1 for the 3MM assay procedure and the synthesis peptide for the cellulose phosphate procedure. Relative activities listed in parentheses for protein kinase NII were obtained with the usual substrate with an additional 1 mg/ml mixed histone.

	ATP						GTP
	Protein kinase NI		Cyclic AMP-dependent kinase		Protein kinase NII		3MM
	3MM	P-81	3MM	P-81	P-81	3MM	
Phosvitin	63	—	0	—	—	100 (55)	100
α-Casein	100	—	0	—	—	19 (11)	33
H1	10	4	100	50	8	3 (2)	3
Mixed histone	2	0	48	37	3	1 (1)	1
H2a	0	1	21	24	1	1 (1)	1
H2b	3	3	0	13	14	2 (4)	1
H3	0	0	13	13	1	2 (2)	0
Protamine	0	4	19	75	9	2 (3)	1
Kemptide	—	2	2	100	0	—	—

preference for exogenous substrates is essentially the same whether ATP or GTP is utilized as phosphate donor.

This apparent order of substrate preference by protein kinase NII is altered when mixed histone is added to assays containing acidic substrates. The presence of histone suppresses the incorporation of P_i into exogenously added acidic proteins while incorporation into exogenously added basic proteins is either slightly enhanced or unchanged.

Kinetic parameters for substrates, nucleoside triphosphates and divalent cations

The K_m values of protein kinases NI and NII for preferred substrates and nucleoside triphosphates were also determined. It can be seen in Table II that the Michaelis constants of protein kinase NI (0.3 mg/ml and 1.02 mg/ml) and protein kinase NII (0.86 mg/ml and 1.2 mg/ml) for their preferred substrates, phosvitin and casein, are very similar. However, protein kinase NII utilized both ATP and GTP as P_i donor, exhibiting K_m values of 20 μM and 30 μM, respectively; while protein kinase NI also utilized ATP (K_m = 50 μM) efficiently, but GTP (K_m = 1 mM) poorly. The values of K_m and V for Mg^{2+} and Fe^{2+} are also comparable between the two enzymes. However, the V of protein kinases NI and NII are 600-fold and 100-fold greater, respectively, for these two enzymes in the presence of optimal Fe^{2+} over that in the presence of optimal Mg^{2+}.

Requirements for Divalent cations

Table III indicates that ions other than Mg^{2+} may substitute partially or fully for both enzymes depending upon the concentration tested. At concentrations of 1 mM, substantial substitution is displayed by Mn^{2+} (26%), Co^{2+} (25%) and Fe^{2+} (83%) in reactions of protein kinase NI. At this level all cations (especially Ca^{2+} (85%) and Co^{2+} (62%)) exhibit some activity with protein kinase NII. At

TABLE II

KINETIC PROPERTIES OF PROTEIN KINASES NI AND NII FOR PREFERRED SUBSTRATES, NUCLEOSIDE TRIPHOSPHATES, AND DIVALENT CATIONS

50-μl aliquots of protein kinase NI or NII were assayed in the presence of increasing concentrations of protein substrate or nucleoside triphosphate. All assays were conducted at a final concentration of 0.2 M NaCl. Typical Michaelis-Menten saturation curves were transformed by plots of s/v vs. s and extrapolation of values for K_m and V. Experiments were performed with enzyme preparations of various late stages of purification without an apparent effect on kinetic properties. Maximum velocity is expressed as pmol/30 min.

Substrate	Protein kinase			
	NI		NII	
	K_m	V	K_m	V
Phosvitin	0.3 mg/ml		0.86 mg/ml	
Casein	1.02 mg/ml		1.2 mg/ml	
ATP	50 μM	65	20 μM	15
GTP	1 mM	18	30 μM	15
Mg^{2+}	0.5 mM	5	0.5 mM	30
Fe^{2+}	5 mM	$3 \cdot 10^3$	2.5 mM	$2.5 \cdot 10^3$

TABLE III

DIVALENT CATION REQUIREMENTS OF PROTEIN KINASES NI AND NII

50-μl aliquots of the purified enzymes were assayed in the presence of either 1 mM or 10 mM divalent cation. Samples of enzyme were first dialyzed overnight (twice 1 l) against 0.05 M Tris-HCl, 1.0 mM EDTA, 10% glycerol, 0.4 M NaCl. All values are expressed relative to the activity obtained in the presence of Mg^{2+}. Casein and phosvitin substrates were employed for assays of protein kinase NI and NII, respectively.

	Protein kinases			
	NI		NII	
	1 mM	10 mM	1 mM	10 mM
Mg^{2+}	100	100	100	100
Mn^{2+}	26	6	35	4
Ca^{2+}	0	3	85	4
Zn^{2+}	2	2	25	0
Cu^{2+}	0	0	41	0
Co^{2+}	25	6	62	1
Fe^{2+}	83	600	34	1260

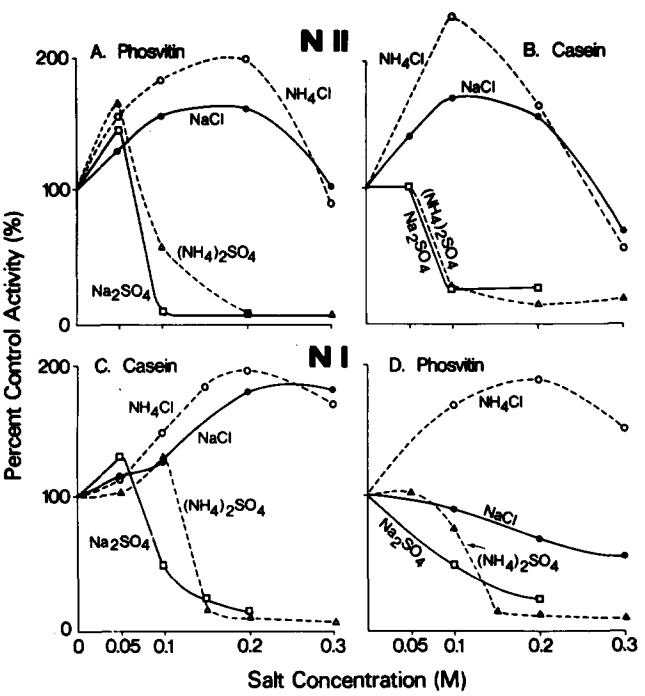

Fig. 2. Effect of various salts on nuclear protein kinase activities. Aliquots of protein kinase NI (50—100-fold purified) and protein kinase NII (100—300-fold purified) were dialyzed against 0.05 M Tris-HCl (pH 7.9), 5.0 mM $MgCl_2$, 1.0 mM EDTA and 10% glycerol until salt was removed as determined by measurements of conductivity. 50-μl aliquots of protein kinase NII were then assayed using (A) phosvitin or (B) casein and protein kinase NI using (C) casein or (D) phosvitin in the presence of various concentrations of salts. ○———○, NH_4Cl; ●———●, NaCl; △———△, $(NH_4)_2SO_4$, and □———□, Na_2SO_4. Reactions were teminated by pipetting 80 μl onto Whatman 3 MM papers and washing with trichloroacetic acid. Values obtained from assays containing exogenous substrate were corrected for endogenous phosphorylation by subtraction of values obtained in the abscence of exogenous substrate.

10-mM concentrations, quite different levels of activity are observed. Most of these divalent cations were inhibitory to both enzymes at this concentration with the exception of Fe^{2+} which produces even higher activity than Mg^{2+}. It was found that Fe^{3+} also gives levels of activity comparable to those with Fe^{2+}.

Effect of salts of monovalent cations

The activity of the two enzymes in the presence of various concentrations of salts of monovalent cations was also determined. These results are seen in Fig. 2. The activity of both enzymes is stimulated by 0.1—0.2 M concentrations of NaCl or NH_4Cl followed by inhibition at higher concentrations. This behavior was observed for protein kinase NII using either casein or phosvitin substrates. However, using phosvitin as substrate, protein kinase NI was not activated by NaCl. Effects of KCl and NaCl on protein kinase NII activity using casein substrate were indistinguishable. Both enzymes are inhibited by $(NH_4)_2SO_4$ and Na_2SO_4 at concentrations greater than 0.1 M. Using their preferred substrates, both enzymes are slightly stimulated by 0.05—0.1 M.

Effect of inhibitors of cyclic nucleotide-dependent protein kinases

The cyclic nucleotide-dependent protein kinases are characterized by inhibition of activity (of the catalytic subunit) by isolated regulatory subunits or preparations of a heat-stable inhibitor. The data of Table IV indicate that under the conditions of these assays, the catalytic subunit of type II enzyme from skeletal muscle is inhibited 55% by rat liver heat-stable inhibitor protein and completely inhibited by either homologous regulatory subunit. In contrast the preparations of nuclear protein kinases are only slightly inhibited by the higher concentrations of regulatory subunits. Inhibition of protein kinases NI and NII activity was also obtained with heat-stable inhibitor, although to a lower level than that obtained for the cytoplasmic enzyme.

TABLE IV

EFFECT OF INHIBITORS OF CYCLIC AMP-DEPENDENT PROTEIN KINASE

Purified protein kinase NI, NII and the catalytic subunit of type II cyclic AMP-dependent protein kinase (CII) from bovine skeletal muscle were tested for inhibition of type I or II regulatory subunits or a heat-stable protein kinase inhibitor preparation from rat liver. Values are expressed as the percentage of activity relative to that obtained in basic assays without other additions. The amount of inhibitors (μg) employed in each assay is indicated in parentheses. Data are presented from experiments in which the minimal molar ratio of RI or RII and heat-stable inhibitor to CII was 5/1 and 500/1, respectively. Molar ratios of regulatory subunits to nuclear protein kinases are in excess of that used for CII. Assays of CII contained hydrolyzed and dephosphorylated casein (19.2 mg/ml) and those for nuclear protein kinases contained α-casein (12 mg/ml). Assays for inhibition by heat-stable inhibitor also contained 10/5 cyclic AMP.

	NI	NII	CII
Regulatory	101 (3.5)	109 (3.5)	0 (3.5)
Subunit I	90 (7.0)	103 (7.0)	1 (4.7)
Regulatory	92 (2.4)	110 (2.4)	1 (4.7)
Subunit II	77 (4.7)	85 (4.7)	
Rat liver	112 (25)	86 (25)	83 (25)
Protein kinase inhibitor	78 (50)	72 (50)	45 (50)

Discussion

This characterization of two cyclic nucleotide-independent protein kinases from rat liver nuclei indicates that these enzymes differ from each other in many ways. The molecular weights of the native enzymes are different [21,22] as are those of the constitutive polypeptide subunits which comprise these enzymes (Fig. 1). Protein kinase NII will utilize both ATP and GTP as phosphate donor while protein kinase NI will only utilize ATP (Table II). At low concentrations of divalent cation (1 mM), partial catalysis by protein kinase NII is supported by all ions tested (relative to Mg^{2+}) while protein kinase NI will only utilize some of them. In addition, when phosvitin is used as phosphate acceptor, NaCl stimulates the activity of protein kinase NII while the activity of protein kinase NI is inhibited by this salt.

However, additional data presented suggest that the two nuclear protein kinases also resemble each other in many of their properties even though they are clearly different enzymes. These similarities include the majority of the effects of monovalent cations, Michaelis constants for casein, phosvitin, and ATP (Table II), preference for exogenous acidic protein phosvitin and casein (Table I) and inhibition at high (10 mM) levels of divalent cations (Table III). The only divalent cations which were not inhibitory at the higher concentration were Mg^{2+} and Fe^{2+}. In fact, at 10 mM, Fe^{2+} stimulated protein kinases NI and NII six and 12-fold, respectively, above that observed with 10 mM Mg^{2+}. The calculated V for protein kinases NI and NII are significantly higher than those obtained in the presence of Mg^{2+}. This result may suggest that Fe^{2+} is a preferred cation for the nuclear protein kinases. Stern et al. [27] have reported that a significant amount of non-heme iron is present in isolated nuclei but the significance of this observation is unknown. The polyamines spermine and spermidine were also tested for their ability to substitute for Mg^{2+}. At 1 mM, these compounds were equally effective, but at 10 mM they were inhibitors as are most other divalent cations. Protein kinase NII was strongly inhibited (77%) by 10 mM spermidine.

Evidence is also presented to indicate that the nuclear protein kinases NI and NII are different from the cytoplasmic cyclic nucleotide-dependent protein kinases. Protein kinases NI and NII are not stimulated by cyclic nucleotides [21,22] and prefer the acidic substrates casein and phosvitin, respectively. In contrast, the cyclic AMP-dependent cytoplasmic enzymes preferentially phosphorylate the basic proteins histones and protamine. While this study does not examine the endogenous nuclear proteins as substrates, preliminary studies suggest that the preferred endogenous substrates (at least in the case of protein kinase NII) are acidic proteins (Thornburg, W., Kieras, R., and Lindell, T.J., unpublished data).

Although some inhibition of both protein kinases NI and NII is obtained with the pure type I and type II regulatory subunits, the effect on the activity of the nuclear protein kinases is not nearly as significant as the inhibition of the skeletal muscle enzyme (Table IV). Partial inhibition of the nuclear enzymes is also seen after addition of a heat-stable inhibitor prepared from rat liver. However, on a weight basis, a large excess of this preparation (greater than or equal to 10^3) is necessary to produce this effect. Therefore, the significance of this effect is questionable.

Additional studies, such as pH optima, sedimentation behavior and the effects of other agents on nuclear protein kinase activity have been investigated by others using impure preparations (see Ref. 9—15 and 19 and 20). During the course of this investigation it was observed that the substrate specity can vary depending on the degree of enzyme purification, especially at the early stages. This is probably due to the presence of addditional protein kinase activities which have recently been found to be present in some preparations (Thornburg, W. and Lindell, T.J., unpublished results). It is, therefore, possible that some earlier studies of impure preparations reflected the behavior of aggregate mixtures of protein kinase activities.

While the total number and types of protein kinases within the nucleus remain to be elucidated, the present characterization of protein kinases NI and NII should be useful as a criterion of comparison when additional enzymes are purified to homogeneity. Since many protein kinases are autophosphorylated [28], including protein kinases NI and NII (Thornburg, W. and Lindell, T.J., unpublished results), it becomes important to know the ultimate subunit structure prior to investigations of the endogenous substrates.

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