Characterization of a Cyclic Nucleotide-independent Protein Kinase Highly Active in Human Adrenocortical Carcinoma

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ABSTRACT

Study of the protein kinase activity pattern of four human adrenocortical carcinoma showed that in all the samples examined a histone kinase (HK III) activity was present at high level, whereas it was barely detectable in normal tissue. HK III was separated from other known adrenocortical protein kinases by diethylaminoethyl cellulose chromatography. Isolated HK III exhibited a histone (H2B) protamine-phosphotransferase selectivity and used adenosine triphosphate but not guanosine triphosphate as phosphate donor. Serine was identified as the only target amino acid phosphorylated in the protein substrate. HK III showed an apparent molecular weight of 65,000 upon gel filtration and an apparent sedimentation coefficient of 3.7S. HK III activity was cyclic adenosine 3':5'-monophosphate independent and was not influenced by calcium, calmodulin, polyamines, and heparin. The significance of HK III activity in adrenocortical carcinoma extracts at a high level as compared to that of normal tissue remains to be clarified with regard both to its possible relationship with tumoral cell growth and differentiation processes and to its potential interest as a marker of human tumoral tissue activity.

INTRODUCTION

Phosphorylation-dephosphorylations of proteins have been recognized for some time as an important regulatory mechanism of cellular functions (6, 27). Protein kinases occur in a variety of enzymatic forms exhibiting different subcellular distribution and various substrate specificities (19, 28, 31). These enzyme activities may be classified according to their specific intracellular effector(s) (19). Qualitative and quantitative differences of the protein kinase pattern among various cell types may determine the behavior of the cell toward a particular effector such as a cyclic nucleotide (6). cAMP-dependent as well as AMP-independent protein kinase activities have been suggested to be related to the control processes of normal cell growth, and specific phosphoproteins have been described in relation to cell growth promotion (16, 21, 26) or cell growth arrest (18). Specific growth factors have been shown to rapidly stimulate a particular type of protein kinase activity in their target cells (2). Possible relevance of protein kinase activities with cell transformation and cancer has been suggested recently by the demonstration that the product of viral genes associated with cell transformation possesses a cyclic nucleotide-independent protein kinase activity (7, 10).

The adrenal cortex contains several types of soluble protein kinase activities. In addition to the well-characterized, hormonally regulated cAMP-dependent system represented by 2 isoenzymes (14), we have previously characterized several cyclic nucleotide-independent activities in adrenocortical bovine tissue (4, 5). The previously used methodology was applied to the study of the protein kinase activity pattern of human adrenocortical carcinoma. In 4 tumors examined, it was found that a particular protein kinase was present at a high level of activity as compared to that found in human normal adult tissue. This paper reports the major molecular and catalytic properties of this protein kinase, which belongs to the category of cyclic nucleotide-independent histone kinases. Further work is needed to assess whether this protein kinase can be considered as a marker of adrenocortical carcinoma and whether a more general interest can be attributed to this activity with regard to its possible relationship with the processes of normal and pathological cell growth and differentiation.

MATERIALS AND METHODS

[γ-32P]ATP (20 Ci/mmol) and [γ-32P]GTP (20 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, England). Phosphocellulose (PII) and DEAE-cellulose (DE52) were obtained from Whatman (Clifton, N. J.); Sepharose 6B and Sephadex G-50 superfine were from Pharmacia (Uppsala, Sweden). Histone (1, 2A, 2B, and 4), bovine serum albumin (Cohn Fraction V), glycogen synthase, L-1-tosylamide-2-phenylethylchloromethyl ketone, N-p-tosyl-L-lysine chloromethyl ketone, phosphoserine, and phosphoethreonine were purchased from Sigma Chemical Co. (St. Louis, Mo.). Casein (Merck, Darmstadt, West Germany) was treated according to published procedures (1) before use. Purified ox brain calmodulin was a generous gift from Professor J. Demaile. Crude preparations of the bovine muscle protein kinase heat-stable inhibitory protein was obtained by the procedure of Ashby and Walsh (1).

Adrenal Tissue

Preparation of Cytosol. Human adrenal gland (normal and tumoral) from patients undergoing surgery were stored at −196° immediately after withdrawal. All further procedures were performed at 0–4°. The tissue was homogenized in ice-cold TDG buffer containing 0.1 M NaCl, using a Potter-Elvehjem apparatus with a Teflon pestle. The homogenate was centrifuged at 105,000 x g for 45 min at 4° (MSE 50 centrifuge). After centrifugation, the supernatant (cytosol) was used for enzymatic studies. Protein determinations were performed by the method of Lowry et al. (22) using bovine serum albumin as the standard.

Subcellular Fractionation. Adrenal tissue (30 g) was homogenized in 50 ml ice-cold Buffer A (50 mm Tris-HCl, pH 7.4, containing 0.25 M sucrose, 25 mm KCl, and 10 mm MgCl2). The homogenate was centrifuged at 4° for 10 min at 600 x g to yield a crude nuclear pellet.
This pellet was resuspended in Buffer A supplemented with 1.8 M sucrose and centrifuged at 4° for 20 min at 55,000 × g. The resulting pellet was washed with 3 × 10 ml of Buffer A and centrifuged again for 5 min at 600 × g. The final pellet represented the nuclear fraction. The postnuclear supernatant was centrifuged at 4° for 10 min at 1000 × g; the pellet was discarded, and the resulting supernatant was centrifuged at 4° for 20 min at 9000 × g to yield a crude mitochondrial fraction. The postmitochondrial supernatant was centrifuged at 4° for 1 hr at 105,000 × g to yield a microsomal pellet and a high-speed supernatant (cytosol). All the subcellular fractions were resuspended in 7 ml TDG buffer containing 0.5 mM NaCl and 0.5% Nonidet P-40. Before use, each fraction was homogenized with a Dounce homogenizer, incubated at 30° for 10 min, diluted by 45 ml of TDG buffer containing 0.16 M NaCl, and then centrifuged at 15,000 × g for 15 min. The supernatant was used for enzymatic studies.

**Protein Kinase Assay**

This was performed following the previously described conditions (4, 5) using the trichloroacetic acid precipitation procedure described in Ref. 30. The standard reaction mixture (80 µl) contained 0.01 mM [γ-32P]ATP (specific activity, 1000 cpm/nmol) and 750 µg histone or casein. MgCl2 was present at a final concentration of 5 mM for cAMP-dependent protein kinase assay and 50 mM for casein and cAMP-independent histone kinase assay. When present, cAMP was used at 5 µM final concentration in the presence of 6 µM theophylline. The reaction was initiated upon addition of the enzyme preparation and run at 30° under linear conditions with regard to time and protein concentration. 32P incorporation was determined using Aquasol (New England Nuclear) as the scintillation mixture in an Intertechnique SL-32 spectrometer.

**Gel Filtration**

This was performed on Sephadex G-200 superfine columns and apparent molecular weights calculated using catalase (M, 240,000), glucose oxidase (M, 150,000), ovine serum albumin (M, 69,000), and trypsin (M, 25,000) as marker proteins.

**Sucrose Density Gradient Centrifugation**

Sedimentation coefficients were determined (23) by centrifugation at 125,000 × g for 16 hr at 4° in a 5 to 20% linear sucrose density gradient in TDG buffer containing 0.5 M NaCl.

**Identification of Phosphoamino Acids after Protein Phosphorylation**

Following the protein kinase reaction, the trichloroacetic acid precipitate (see above) was subjected to acid hydrolysis after successive washings with 1 ml H2O, 1 ml ethanol:ether (1:4, v/v) containing 0.1 N HCl, and 1 ml ethanol:ether (1:4, v/v). The precipitate was resuspended in 0.1 M formic acid:ethanol (98% by volume) and lyophilized. For acid hydrolysis, the lyophilisate was treated with 6 N HCl at 110° for 2 hr under a nitrogen atmosphere. HCI was removed under reduced pressure, and the hydrolysate was dissolved in a marker mixture containing authentic phosphoserine and phosphothreonine, each at 1 mg/ml. The mixture was analyzed by electrophoresis on cellulose thin-layer plates at pH 1.9 for 2 hr at 1.1 kV in glacial acetic acid:formic acid:H2O (156:60:794, v/v). Marker phosphoamino acids were detected after staining with ninhydrin, and 32P labeling was visualized after autoradiography using Kodak X-O Mat films.

**RESULTS**

**Presence in Adrenocortical Carcinoma Extracts of a Protein Kinase Activity Which Is Barely Detectable in Normal Tissue**

Characterization of the protein kinases present in a tissue extract requires adequate resolution to avoid the overlap between the various types of active moieties. Previous studies with bovine adrenal cortex cytosol had shown that a single ion-exchange chromatography step such as a DEAE-cellulose column was not sufficient to completely resolve the cAMP-dependent histone kinase activity and the cAMP-independent casein kinase systems (5). However, it has been found previously that the cyclic nucleotide-independent casein kinases exhibited a particular affinity for phosphocellulose (4, 5). Normal and tumoral human adrenal cortex cytosol were thus first adsorbed onto a phosphocellulose column (1.5 × 6 cm) previously equilibrated with TDG buffer containing 0.2 M NaCl. As previously found for bovine tissue (4), the washthrough volume of the column contained the bulk of histone kinase activity, whereas casein kinase could be eluted by TDG buffer containing increasing concentration of NaCl and separated into A type and G type (4, 5) casein kinase moieties (not shown). The TDG buffer:0.2 M NaCl phosphocellulose wash was then diluted to 0.04 M NaCl and transferred onto a DE52 cellulose column (1.5 × 6 cm). Elution with TDG buffer containing a linear (0.04 to 0.3 M) NaCl gradient resulted in the protein kinase patterns illustrated in Chart 1. Normal human tissue yielded 2 major peaks of histone kinase activities, eluting at average salt concentrations of 90 and 130 mM NaCl, respectively (Chart 1A). Both peaks contained a cAMP-dependent activity inhibited by the heat-stable cAMP-dependent protein kinase inhibitor of Ashby and Walsh (1). Together with this elution behavior, these properties agree with the fact that Peaks I and II are mostly represented by the well-known cAMP-dependent isoenzymes PK I and PK II (11, 14). In addition, a residual histone kinase activity was detected in the presence of heat-stable cAMP-dependent protein kinase inhibitor in the tail of the PK II peak (Chart 1A). Adrenocortical carcinoma extract (Chart 1B) similarly exhibited 2 histone kinase activities which could be attributed to the PK I and PK II cAMP-dependent isoenzymes. However, the tumoral tissue strikingly differed from its normal counterpart by the presence of an additional histone kinase activity (Chart 1B, Peak III). This Peak III was found in the 4 adrenocortical carcinoma extracts examined; this histone kinase activity was in all cases mostly insensitive to cAMP and not influenced by the heat-stable cAMP-dependent protein kinase inhibitor in the presence of cAMP. It was thus concluded that the adrenocortical tumors examined contained a high level of a cAMP-independent protein kinase activity, which was not detected in normal tissue. A more detailed characterization of this activity was attempted in further experiments.

**Isolation of the cAMP-independent Histone Kinase Activity Found in Adrenocortical Tumor Extract**

In order to resolve the cAMP-independent histone kinase (Chart 1B, Peak III) from the interfering cAMP-dependent PK II (Chart 1, Peak II), the active fractions corresponding to Peak III were diluted 3-fold with TDG buffer containing 10 µM cAMP and incubated at 30° for 10 min in order to fully dissociate into subunits all contaminating cAMP-dependent holoenzyme present in these fractions. The preparation was then loaded onto a 0.9- × 3-cm DEAE-cellulose column previously equilibrated in TDG buffer containing 10 µM cAMP. The column was washed with 3 volumes of the same buffer containing 0.1 M NaCl and then eluted with 4 volumes of TDG:0.1 M NaCl without cAMP. Finally, 3 column volumes of TDG buffer:0.3 M NaCl allowed the recovery of the histone kinase activity, eluting as a single
peak (not shown). This activity was influenced neither by the presence of cAMP nor by the presence of the heat-stable cAMP-dependent protein kinase inhibitor in the presence or absence of cAMP. The preparation was thus devoid of any detectable cAMP-dependent protein kinase holoenzyme, as expected, and also devoid of any detectable cAMP-dependent protein kinase free catalytic subunit. The corresponding fractions did not exhibit any detectable casein kinase activity. These fractions were pooled and used for further experiments in which they will be referred to as the HK III preparation.

Molecular and Catalytic Properties of the Tumoral cAMP-independent Histone Kinase (HK III) Preparation

The histone kinase activity (HK III) chromatographed upon gel filtration through a Sephadex G-200 column as a single symmetrical peak of activity. Calibration of the column with marker proteins, as indicated under "Materials and Methods," allowed calculation of an apparent molecular weight of 65,000. Velocity sedimentation study in sucrose density gradient yielded a single peak of histone kinase activity exhibiting a calculated sedimentation coefficient of 3.7S.

Substrate Specificity. A number of known phosphate acceptor proteins were tested as potential substrates for HK III activity. The results collected in Table 1 confirmed that the enzyme belongs to the histone kinase type with negligible activity toward casein, whereas protamine was also a good phosphate acceptor. Phosphorylase b and glycogen synthase were poor substrates for the HK III preparation. Among the various histones available, histone H2B appeared by far the preferred substrate, H2A and H1 being much less actively phosphorylated whereas H4 was negligibly labeled. In order to determine the target amino acids of HK III in the phosphorylated protein substrate, histone H2B was labeled with $^{32}$P in the presence of $[^{33}P]ATP$ and HK III and thereafter subjected to HCl hydrolysis. The resulting amino acid mixture was analyzed by electrophoresis as indicated under "Materials and Methods." As shown in Fig. 1, autoradiography disclosed that the only detectable labeled amino acid corresponded to phosphoserine whereas no phosphothreonine or phosphotyrosine could be detected.

Kinetic Properties. Kinetic properties of HK III were examined using histone H2B and ATP as substrates. The protein phosphorylation rate was linear for 10 min at 30° and up to 70 $\mu$g of protein in the assay. Varying ATP or histone H2B concentrations allowed graphical calculation using Lineweaver-Burk plots with apparent $K_m$ values of 14.8 $\mu$M for ATP and 158 $\mu$g/ml for histone H2B. HK III appeared to be unable to use $[^{32}P]GTP$ as the phosphate source.

Effect of pH, Ions, and Various Effectors on HK III Activity.

Using histone H2B as the protein substrate, HK III was found to be inactivated below pH 6 and above pH 10, the maximal activity being observed at an optimal pH of 8 to 8.5.

The presence of Mg$^{2+}$ was required for HK III activity, which was stimulated by increasing Mg$^{2+}$ concentration to reach a plateau at about 30 mM Mg$^{2+}$. From this result, HK III activity was thus routinely assayed at 50 mM Mg$^{2+}$ concentration. Ca$^{2+}$ and Zn$^{2+}$ could not substitute for Mg$^{2+}$ in the reaction, whereas Mn$^{2+}$ favored the reaction up to 2.5 to 3 mM, higher concentrations being inhibitory. Increasing NaCl concentration in the assay medium (containing 50 mM Mg$^{2+}$) resulted in a limited apparent (10%) stimulation of HK III activity up to 100 mM and an inhibitory effect above this concentration. The enzyme activity was totally inhibited above 500 mM NaCl concentration in the assay.
Adrenocortical Carcinoma Protein Kinases

Typical effectors of other types of protein kinase activities, namely, cAMP, cyclic guanosine 3'-5'-monophosphate, heat-stable cAMP-dependent protein kinase inhibitor (as already shown), as well as calmodulin in the presence of calcium, polyamines, and heparin were without any effect on HK III activity as measured using histone H2B as the protein substrate, under the optimal incubation conditions defined above.

Subcellular Distribution of the cAMP-independent Histone Kinase HK III in Normal and Tumoral Tissues

Both an adrenocortical tumor homogenate and a sample of normal tissue were processed as described under "Materials and Methods" to yield nuclear, mitochondrial, microsomal, and cytosolic fractions. After a 5-fold dilution in TDG buffer, each fraction was subjected to phosphocellulose adsorption followed by a DEAE-cellulose chromatography as aforementioned for the isolation of the cytosolic cAMP-independent histone kinase activity. Assay of HK III with histone H2B as substrate in the different enzyme preparations yielded the data illustrated in Table 2. It can be seen that, while the highest HK III specific activity was found in the microsomal fraction, the bulk of the total enzyme activity was recovered as a soluble moiety in the cytosol fraction. A general increase of HK III activity in all subcellular fractions was observed in carcinoma tissue, together with an elevation of the enzymatic specific activity, specially marked in mitochondrial and microsomal compartments.

DISCUSSION

Study of the protein kinase pattern of 4 human adrenocortical carcinoma disclosed in all tumor samples examined the presence of a protein (histone) kinase activity, which was barely detectable in normal tissue. On the basis of its functional as well as molecular properties, this cyclic nucleotide-independent histone kinase activity appeared clearly different from the 2 cAMP-dependent protein kinase holoenzymes known in this tissue and also different from the free catalytic subunit of these holoenzymes (11, 14). Partial purification of the enzyme activity yielded a preparation which exhibited a histone (protamine) kinase specificity and used only ATP as a phosphate source. Serine was the only detected target amino acid in the protein substrate. Due to its elution behavior from a DEAE-cellulose resin, this tumor-characteristic histone kinase was termed HK III. In addition to its cyclic nucleotide-independent nature, HK

Table 2
Subcellular distribution of HK III activity in human normal and adrenocortical carcinoma tissues

<table>
<thead>
<tr>
<th>Subcellular preparation</th>
<th>Cytosol</th>
<th>Microsomes</th>
<th>Mitochondria</th>
<th>Nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Activity</td>
<td>Specific Activity</td>
<td>Total Activity</td>
<td>Specific Activity</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>12,700</td>
<td>14</td>
<td>820</td>
<td>12.5</td>
</tr>
<tr>
<td>Carcinoma</td>
<td>26,570</td>
<td>24</td>
<td>2,730</td>
<td>107</td>
</tr>
</tbody>
</table>

* Total HK III activity recovered in the corresponding fraction, expressed as pmol 32P incorporated per min.

* Specific HK III activity of the given fraction, calculated as pmol 32P incorporated per min per mg protein.
Ill activity was not influenced by calcium:calmodulin, polyamines, or heparin, which are known effectors of other type of protein kinases (13, 17, 19). Due to these functional characters, HK III may be classified as a messenger-independent protein kinase (19). HK III exhibited an apparent molecular weight of 65,000, clearly different from those of other messenger-independent protein (casein) kinases found in bovine adrenocortical tissue (4, 9). HK III appeared also different from the pp60⁰⁰⁰⁰ protein kinase occurring in virally transformed cells; pp60⁰⁰⁰⁰ prefers casein as exogenous protein substrate and can use GTP as well as ATP as phosphate donor (7, 10). In addition, the gene product of viral transformation phosphorylates tyrosyl residues in protein substrates (7), which is not the case with HK III.

Although adrenocortical HK III exhibits a different apparent molecular size, it presents some functional similarities with the cAMP-independent histone kinases described in brain, thymus, and lymphocytes and classified as protein kinase c by Weller (31). More recently, a cAMP-independent histone kinase has been found at high level in acute myeloblastic leukemia cells, as compared to normal myeloid tissue (29). This kinase exhibits a slightly lower apparent molecular weight (M, 48,000) than did our adrenocortical HK III. On the other hand, Lake (21) has described in Chinese hamster cells a cAMP-independent histone kinase, which might be related to cellular growth activity. However, this enzyme was strongly associated with chromatin and required 0.35 M NaCl to be extracted from nuclei, which is obviously not the case with HK III. In addition, lysine-rich histone H1 was the best substrate for this nuclear protein kinase (21), whereas HK III better phosphorylates histone H2A and H2B.

The relevance of the high level of HK III activity in adrenocortical carcinoma as compared to normal tissue with regard to the malignant state remains to be established. In recent years, cellular protein phosphotransferase activities have been suggested to be related to the normal and/or pathological cell proliferation processes (3, 8, 12, 26, 28, 31). A particular interest has been focused on the study of changes in cAMP-dependent protein kinase activities or holoenzyme ratio in relation to normal or pathological cell growth (2, 12, 26, 29, 31). Nuclear histone kinase activities have been found elevated in actively regenerating tissues (12, 26), and during fetal and neonatal development (26). This type of activity has been correlated with the regression of estrogen-dependent mammary tumors in humans (3). However, in adrenocortical carcinoma, HK III has been found to be mostly associated with the cytosol fraction. In human adrenal cortex tumors of various types, Riou et al. (27) reported the lack of a cAMP-dependent holoenzyme (PK II) in a case of an adrenocorticotropic hormone-insensitive tumor, but no additional protein kinase activity was mentioned in this study. On the other hand, mutants of a mouse adrenal tumor cell line exhibiting an impaired cAMP-dependent protein kinase system have been characterized (15).

However, to our knowledge, an HK III-type activity has not yet been recognized in adrenocortical tissue. In toxic adenoma thyroid tissue, Munari et al. (24, 25) have characterized a cAMP-independent histone kinase activity which was not found in normal tissue extracts. This enzyme exhibited several functional properties similar to those of our adrenal HK III. This observation, together with the presence of a rather similar cAMP-independent histone kinase in acute myeloblastic leukemia cells (9), suggests that this type of phosphotransferase activity may be of interest to study in pathological cases in which the normal control of cell proliferation and growth processes are altered.

On the other hand, considering the differentiated functions of adrenal cortex tissue, it remains to be seen whether HK III may be involved in some way in the control of the corticosteroidogenetic pathway. This point would be of interest in secreting adrenocortical carcinoma, especially in those cases where steroidogenesis is not responsive to normal stimuli such as cAMP and adrenocorticotropin hormone (27).

It will be of interest to examine whether protein kinase activity of the HK III type is a general characteristic of the tumoral state in other tissues. This approach is in progress in the laboratory in the case of human mammary tumors. In addition to the fact that HK III-type activities may be concerned with some fundamental role in cell metabolism, its potential interest as a possible marker of the tumoral state and/or of the tumoral evolution appears worth investigating.

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REFERENCES


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