Evidence for the Presence of Protein Kinases Which Stimulate Phosphorylation of c-erb A Protein in Rat Kidney Nuclei

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Abstract. Protein kinases were separated from rat kidney nuclear extract by hydroxylapatite column chromatography. Five (I-V) different protein kinases were isolated when histone was used as a substrate. Two (I and III) of them stimulated phosphorylation of c-erb A-β protein (50 kDa) expressed in Escherichia coli. The c-erb A product has an activity of high affinity T₃ binding. One (I) of the kinases was dependent on cyclic adenosine 3', 5'-monophosphate (cyclic AMP). The other kinase (III) was not dependent on cyclic nucleotides. The latter kinase was eluted from hydroxylapatite column with 0.05 M PO₄ at pH 7.4. The sedimentation coefficient(s) estimated by continuous sucrose density gradient centrifugation was approximately 6.0. Km values for ATP were estimated by double reciprocal analyses, which gave 110.0 μM in the protein kinase I (in the presence of 10⁻⁶M cyclic AMP) and 25 μM in the protein kinase III, respectively. The data showed that 1.0 mol phosphate was incorporated into 80 mol of c-erb A protein (50 kDa) either in the presence of protein kinase I (with 10⁻⁶M cyclic AMP) or in the presence of protein kinase III. These results suggested that there are protein kinases for c-erb A protein, whose functional properties are similar to those of nuclear T₃ receptor, in rat kidney nuclei.

IT IS WELL documented that mammalian genomes contain multiple c-erb A-β related genes [1, 2]. Some of them encode high affinity T₃ receptors [2–5]. T₃ receptors mediate hormone action by modulating the transcription of specific genes. This process requires receptors to bind to regulatory DNA sequence elements located close to the target genes [6–9]. It has also been demonstrated that steroid hormone receptors which have functional properties similar to those of thyroid hormone receptors [10, 11] could be phosphorylated, although the role of the phosphorylation events has not been thoroughly clarified [12, 13]. Recently, it has been reported that c-erb A product was also phosphorylated under stimulation of cyclic adenosine 3', 5'-monophosphate (cyclic AMP)-dependent protein kinase or of protein kinase C [14]. However, it is not certain whether thyroid hormone receptors are phosphorylated through the activation of protein kinase present in the nucleus or not. In this study, we aimed to identify the kinase(s) which phosphorylates c-erb A protein in rat kidney nuclei.

Materials and Methods

Materials

DNA restriction, ligation, and electrophoresis were performed as described by Maniatis et al. [15]. 3,5,3'-Triiodo-L-thyronine (L-T₃), adenosine 3', 5'-monophosphate (cyclic AMP), adenosine 5'-triphosphate, isobutylmethyl-xanthine, histone, casein, and bovine serum albumin (BSA) (Fraction V) were obtained from Sigma Chemical Co. (St. Louis, MO). Phenylmethylsulfonyl fluoride (PMSF), sodium dodecyl sulfate (SDS) and dithiothreitol (DTT) were from Nakarai Chemical Co. (Kyoto, Japan). Isopropyl-B-D-thiogalacto-
pyranoside was purchased from Wako Chemical Co. (Osaka, Japan). E. coli-expression plasmid pKK233–2, double stranded calf thymus DNA-cellulose, Sephacryl S-200, Sephacryl S-300, mono Q-Sepharose and protein A were from Pharmacia Fine Chemicals Inc. (Piskataway, NJ). Restriction enzymes were from Takara Shuzou Co. (Tokyo, Japan). Extracti-gel D and reagents for SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Dowex 1-X8, Cl**, 200–400 mesh, anion exchange resin were obtained from Bio-Rad (Richmond, CA). X-ray film (XAR-5) and developing solutions were from Eastman Kodak (Rochester NY). Intensifying screens were from Fuji Film (Tokyo, Japan). [125I]Triiodo-L-thyronine ([125I]T₃) (3000 μCi/μg) and γ-[32P]adenosine 5'-triphosphate (γ-[32P]ATP (100-μCi/nmol) were purchased from New England Nuclear (Boston, MA).

**Construction of plasmid and Preparation of c-erb A protein**

Construction of plasmid, pNTR which expresses human c-erb A protein in E. coli, and the preparation of c-erb A protein was performed as previously described [5]. In this plasmid, the entire coding region of human c-erb A [2] is located in the blunt-ended Ncol site of pKK233-2. This construction located the first methionine codon of the translated region of human c-erb A cDNA at 13 bases downstream of the lacZ ribosome binding sequence (AGGA) of pKK233–2 and produced a non-fusion c-erb A protein. Additionally, in order to increase the yield of the protein, the DNA sequence containing the c-erb A DNA and promoter region was transferred to a pUC plasmid, which has an extremely high copy number in E. coli. After propagation of E. coli (JM103) that were transformed with pNTR, the expression of c-erb A was induced by isopropyl-B-D-thiogalactopyranoside without glucose. After 14 h incubation, E. coli collected by centrifugation was solubilized by sonication and freezing.

**Preparation of c-erb A protein**

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**Preparation of anti-c-erb A antibody**

c-erb A expressed in E. coli was partially purified by sequential fractionation with hydroxylapatite, mono Q-Sepharose and Sephacryl S-200 or S-300 column chromatography and was localized in SDS-PAGE [5, 16]. A protein band of synthesized c-erb A product (50 kDa) was excised from the gel and was eluted from the gel by electrophoresis. The final product was immunized to rabbit. The antibody (γ-globulin fraction) was partially purified by DEAE-cellulose column chromatography and by precipitation with ammonium sulfate.

**Preparation of protein kinases from rat kidney nuclei**

Nuclear fraction was prepared from 150 g male Wistar rats (10 animals) as previously described using discontinuous sucrose density gradient centrifugation [17, 18]. The isolated nuclei showed a protein/DNA ratio of 2.7 and DNA recovery of 60–70%. Phase contrast microscopic observations revealed intact nuclei with minimal cytoplasmic contamination. The nuclei were washed with 10 mM Tris-HCl pH 7.5, containing 0.32 M sucrose and 5.0 mM MgCl₂ three times in order to remove extranuclear components. The extract which was prepared by incubation of the nuclei with 10 mM potassium phosphate buffer pH 7.4, containing 0.4 M KCl, 5.0 mM MgCl₂ (KMP) was applied to a column (1.0 x 10.0 cm) of hydroxylapatite. After washing the gel thoroughly with KMP, proteins were eluted with a 200 ml linear gradient of sodium phosphate buffer pH 7.4 from 10.0 to 100.0 mM.

**Estimation of molecular weight (s value) of protein kinase**

Each protein kinase activity separated by hydroxylapatite column chromatography was applied to a continuous sucrose density gradient (5–20%). The gradient was prepared in 10.0 mM sodium phosphate buffer pH 7.4, containing 0.2 M NaCl, 3.0 mM MgCl₂, 0.5 mM DTT, and 0.5 mM EGTA. After applying each protein kinase (100 μl) onto the top of the gradient, the tubes (4.8ml each) were centrifuged at 50,000 rpm for 12 h at 2°C in an sw 60 rotor (Beckman Ultracentrifuge). External molecular weight markers were simultaneously sedimented as the standard for the evaluation of the sedimentation coefficient as previously described [17, 18].

**[125I]T₃ binding assay**

Assay of binding of [125I]T₃ to the c-erb A protein was performed as previously described [19]. Dowex anion-exchange resin was used for the separation of bound [125I]T₃ from free [125I]T₃ [20]. The specific binding was defined as total [125I]T₃ binding (in the absence of unlabeled T₃) minus the amount of [125I]T₃ bound in the
presence of $10^{-6}$M unlabeled T3.

**Protein kinase assay**

Protein kinase activities isolated from rat kidney nuclei were measured by the method as previously described [21]. Histone (5 µg/tube) or casein (15 µg/tube) were used as the substrate for the kinases. The enzyme fraction (100 µl) was incubated with radioactive ATP (1.5×10^4 cpm added) in a final volume of 150 µl of incubation medium (20 mM Tris-HCl, pH 7.4, containing 3.0 mM MgCl₂, 0.5 mM DTT, 0.5 mM EGTA and 10.0 mM NaF (TMDE) for 5 min at 22°C. The final concentration of ATP (including radioactive ATP) was adjusted to $10^{-5}$M by adding unlabeled ATP. In several experiments, $10^{-6}$M cyclic AMP and 1.0 mM isobutylmethyl xanthine were added. The reaction was terminated by adding 10.0% trichloroacetic acid (TCA) (37.5 µl). After addition of 50 µl of 0.63% BSA, the mixture was vortexed for a short time (approximately 2.0 sec) and TCA-insoluble material was precipitated by centrifugation. The precipitate was washed with 2.5% TCA twice and the final precipitate was dissolved with 50 µl of 90.0% formic acid, then the dissolved material was transferred to a counting vial which contained scintillation liquid (4.0 ml), and radioactivity was measured by Liquid Scintillation Spectrometer (Packard Instrument Co., Downers Grove, IL).

**Phosphorylation of c-erb A protein, SDS-polyacrylamide gel electrophoresis and autoradiography**

$c$-erb A protein-containing fraction (E. coli extract) (100–200 µg protein) was incubated with an appropriate amount of protein kinase(s) in the presence of γ-[³²P]ATP (1.0 µCi) for 5 min at 22°C in a final volume of 150 µl TMDE. The final concentration of ATP (including radioactive ATP) was adjusted to $10^{-5}$M by adding unlabeled ATP. The reaction was terminated by boiling the mixture for 5 min after the addition of Laemmli’s sample buffer with 100 mM DTT. Electrophoresis of the phosphorylated proteins was performed in the presence of 0.1% SDS by the method of Laemmli [22]. The samples were separated on a 1.5 mm thick 10% polyacrylamide slab gel. Gels were stained with Coomasie Brilliant R-250 Blue, destained, dried and exposed to X-ray films at -70°C. The amount of [³²P] incorporated into proteins was quantitated by the method employing the Cerenkov effect of the protein band excised.

**Immunoprecipitation of c-erb A protein**

The phosphorylated samples were incubated with Laemmli’s sample buffer (50 µl) for 30 min at room temperature. After the incubation, each sample was passed through a column (0.5 × 0.5 cm) of Extracti-gel D to remove SDS. The eluted material was incubated with 50 µl of anti c-erb A antibody (diluted to 1:10,000) for 12 h at 10°C. After the incubation, the immune-complexes were separated using protein A adsorption. The complexes were boiled after the addition of Laemmli’s sample buffer with 100 mM DTT and were applied to an SDS-PAGE. Radioactive phosphoproteins developed on the gel were analyzed by autoradiography.

**Protein concentration measurement**

Protein concentration was measured by the method of Lowry et al. [23] with BSA as the standard. The protein concentration of c-erb A band developed on the SDS-PAGE gel was estimated with a densitometer (Hitachi, Tokyo, Japan).

**Results**

**Characteristics of [¹²⁵I]T₃ binding to the c-erb A protein expressed in Escherichia Coli**

As previously described, the 50 kDa product of c-erb A bound T₃ with an affinity constant of 4.3±0.6×10⁹M⁻¹ (mean±SD for 5 experiments). The order of affinity for iodothyronine analogues was triiodothyroacetic acid > 3,5,3'-triiodo-L-thyronine > 3,5,3'-triiodo-D-thyronine > L-thyroxine, and [¹²⁵I]T₃-c-erb A protein complex bound to DNA celluose [5].

**Isolation of protein kinases from rat kidney**

By hydroxylapatite column chromatography of rat kidney nuclear extract, 5 different protein kinase activities (I-V) were separated when histone (whole histone) was used as a substrate (Fig. 1). When casein was used as a substrate, protein kinases I and II were detected (data not shown). The activity of protein kinase I was dependent on cyclic AMP. [¹²⁵I]T₃ binding activity (nuclear T₃ receptor) was eluted with 0.22–0.28 M sodium...
Fig. 1. Isolation of protein kinases from rat kidney nuclei. Nuclear extract obtained from rat kidney was applied to a column of hydroxylapatite, and proteins were eluted with 200 ml of linear gradient of sodium phosphate buffer (pH 7.4) as described under "Materials and Methods". Protein kinase activity was measured using Histone as a substrate in the absence (●) or presence (○) of 10⁻⁶ M cyclic AMP. [¹²⁵I]T₃ binding activity was also measured in each fraction (x), in which [¹²⁵I]T₃ (150,000 cpm added) was incubated with 100 μl aliquots in the presence of 0.5 mM DTT for 4 h at room temperature. Specific binding is illustrated.

phosphate. The elution position of the receptor was between those of protein kinases I and II.

Phosphorylation of c-erb A protein

c-erb A protein (50 kDa) was phosphorylated in the presence of protein kinase I or III. As is shown in Fig. 2, the potency of the enzyme activity was higher in protein kinase III than in protein kinase I. The stimulation of phosphorylation by protein kinase I was only observed in the presence of cyclic AMP. However, kinase III was not dependent on cyclic AMP or cyclic GMP (data not shown). Phosphorylation of this 50 kDa protein was not observed when whole E. coli (c-erb A was not expressed) extract was incubated with protein kinase III or kinase I in the presence of cyclic AMP. The phosphorylation of c-erb A protein by kinase III was observed only in the presence of cyclic AMP. However, kinase III was not dependent on cyclic AMP or cyclic GMP (data not shown). Phosphorylation of this 50 kDa protein was not observed when whole E. coli (c-erb A was not expressed) extract was incubated with protein kinase III or kinase I in the presence of cyclic AMP. The phosphorylation of c-erb A protein by kinase III was observed in its concentration dependent fashion (Fig. 3, lane C to H). The phosphorylated c-erb A protein which was generated by incubation with a minimal concentration of protein kinase III (1.25 μg protein) was concentrated by immunoprecipitation with anti c-erb A antibody (Fig. 4). Phosphorylated protein band which corresponded to the c-erb A protein was not observed when whole E. coli (c-erb A was not expressed) extract was incubated with the kinase (Fig. 3, lanes A and B). Fifty-five kDa protein which was one of the components of E. coli was also phosphorylated in the presence of kinase III in proportion to the concentration of the enzyme. However, the phosphorylation of 55 kDa protein was very low in the absence of c-erb A protein even though incubation was performed with a maximal concentration (15.0 μg protein) of kinase III. When protein kinase III alone was incubated under the same condition as the phosphorylation reaction, no phosphorylated protein band was observed (data not shown). A study with sucrose density gradient centrifugation of protein kinase III showed that the kinase was 6.0s of sedimentation coefficient (Fig. 5). In this study, a large amount of protein kinase activity was sedimented to the bottom of the tube, suggesting that protein-protein interaction among proteins might have occurred. As shown in Fig. 5, phosphorylated material (Band X) which was one of the components of E. coli was also phosphorylated. The s value for maximal phosphorylation, however, was different from that of protein kinase III, indicating that the fraction which contained protein kinase III contained kinase(s) distinct from protein kinase III.

Protein kinase I was also applied to sucrose density gradient centrifugation. The kinase activ-
Fig. 2. Phosphorylation of c-erb A protein. Protein kinases I, III, IV and V were obtained as shown in Fig. 1. c-erb A protein-containing fraction (200 µg protein) which was obtained by solubilization of E. coli that expressed c-erb A was incubated with [32P]ATP in the presence of 25 µg protein of protein kinase I, III, IV or V. After the reaction, phosphorylated proteins were analyzed by autoradiography of the proteins separated on SDS-PAGE. Phosphorylation reaction was performed in the presence of 10^{-6} M cyclic AMP in the case of stimulation with protein kinase I. Similar results were obtained with 2 different lines of solubilized fraction prepared from E. coli which expressed c-erb A.

Fig. 3. Effect of various concentrations of protein kinase III on the phosphorylation of c-erb A protein. c-erb A protein-containing E. coli extract (200 µg protein) (lanes C-H) or extract of control E. coli which did not express c-erb A (lanes A and B) was incubated in the absence (lanes A and C) or presence of 1.25 µg (D), 2.5 µg (E), 5.0 µg (F), 10.0 µg (G) or 15.0 µg (H) of protein kinase III with [32P]ATP and unlabeled ATP as described under “Materials and Methods”. After incubation the materials were applied to SDS-PAGE. The upper panel shows the protein bands stained with Coomassie Brilliant R-250 Blue, and lower panel shows autoradiography of the phosphorylated proteins.

Discussion

We previously demonstrated that cyclic AMP-dependent protein kinase prepared from rat liver stimulated the phosphorylation of c-erb A protein in vitro [24]. Goldberg and his coworkers demonstrated that the phosphorylation of c-erb A product was enhanced 10-fold by treatment of the avian erythroblastosis virus-transformed cells with activators of either protein kinase C or cyclic AMP-dependent protein kinase [14]. We observed that cyclic AMP-dependent protein kinase, which was present in rat kidney nuclei, phosphorylated the c-erb A protein. Thus, the cyclic AMP-dependent kinases, both extra- and intranuclear...
kinases, are able to stimulate phosphorylation of c-erb A protein. In addition to the cyclic AMP-dependent kinase, 6.0s protein kinase which was not dependent on cyclic nucleotides stimulated phosphorylation of c-erb A protein. The Km value was lower in this kinase than in cyclic AMP-dependent protein kinase in nuclei, suggesting that the 6.0s kinase is one of the main kinases for c-erb A phosphorylation. When casein was used as a substrate for protein kinases, the 6.0s enzyme did not phosphorylate the substrate. In view of this finding, it was speculated that substrate specificity might be present in this enzyme. As demonstrated by Goldberg et al. [14], serine residues in c-erb A protein were phosphorylated by stimulating cyclic AMP-dependent protein kinase or protein kinase C, and one of these sites is located in the amino-terminal domain of the molecule. In our study, however, we could not determine the site of phosphorylation in c-erb A protein since the amount of phosphorylated protein was not large enough to analyze the site of phosphorylation. We could detect the two nuclear protein kinases which phosphorylate c-erb A product. The results suggested that there might be several kinases which are distinct from these kinases.

During our studies, we observed that c-erb A protein expressed in E. coli enhanced the phosphorylation of 55 kDa protein which was one of the components of E. coli (Fig. 4). We could not characterize the precise mechanism of c-erb A product-induced augmentation of phosphorylation. However, the fact that the enhancement was...
only observed in the presence of protein kinase III indicated that the c-erb A product has the potency to activate the enzyme.

Analysis of stoichiometry of c-erb A protein phosphorylation showed that it was low (1:80). The precise reason why the value was low could not be solved. A certain mechanism, such as the interaction of the enzyme with certain molecule(s), might be required to complete activation of the enzyme.

Steroid hormone receptors, whose structural and functional properties are closely related to those of thyroid hormone receptors, were reported to be phosphorylated. Although the role of these phosphorylation events has not been thoroughly clarified, the phosphorylation appeared to correlate with the hormone-binding capacity of the receptor [12] or with hormone-induced transformation of the receptor to a high nuclear affinity state [13]. While the role of phosphorylation of c-erb A protein by nuclear protein kinase is no doubt important, the action of the phosphorylated c-erb A protein remains to be elucidated.

References

9. Glass CK, Franco R, Weinberger C, Albert VR,


