Radioimmunoassay of the Regulatory Subunit of Type I cAMP-dependent Protein Kinase*

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A sensitive and specific radioimmunoassay is described which can detect 300 pg of bovine skeletal muscle type I regulatory subunit (R'). Specific R' antisera were raised in rabbits by repeated immunization with R'. Bovine skeletal muscle catalytic subunit (C), bovine heart muscle type II regulatory subunit, and bovine lung cyclic GMP-dependent protein kinase exhibited 0.0025, 0.030, and 0.13% competitive cross-reactivity in the assay.

Bovine tissues contain regulatory subunit which is immunologically identical with those found in pure bovine skeletal muscle protein kinase; however, the degree of immunocross-reactivity differed for other tissues. Rat tissues contain enzyme that cross-react immunologically in a nonparallel fashion with pure bovine skeletal muscle protein kinase, suggesting that partial but not complete identity of antigenic determinants exists between R' of different species.

Among the five antisera screened, one antiserum detected a site-specific, concentration-dependent decrease in R' immunoreactivity as cAMP was varied in the physiological range. cGMP up to 10 μM concentration did not produce any specific effect. C combined with R' also produced a decrease in the immunoreactivity of R'. These results suggest that specific binding of cAMP or C to R' either blocks the R' antigenic determinant or causes a change in the conformation of the molecule which affects the structure of the determinant. It seems possible that this antiserum may be useful as a molecular probe to explore the structure, function, and regulation of type I cAMP-dependent protein kinases.

During the last decade, evidence has accumulated suggesting that the mechanism of action of cAMP in eukaryotic tissues is mediated through cAMP-dependent protein kinases (1, 2). While many forms of protein kinases have been reported, at least two cAMP-dependent protein kinase isozyme forms have been identified in various mammalian tissues. These have been designated as type I and type II based on their elution on DEAE-cellulose by salt gradients (1-5). In all the eukaryotic tissues examined so far, R' of type I and type II cAMP-dependent protein kinases differs with respect to several physicochemical (2, 5-11) and immunological properties (12-14) while C appears similar (9, 15-17). Both of these enzymes are tetramers consisting of cAMP binding dimeric R and two C. Activation of the enzyme occurs by a cAMP-promoted dissociation of the holoenzyme to yield two active C and a dimeric R-cAMP complex (2, 6, 7, 15).

Fleischer et al. (13) have previously reported the development of a radioimmunoassay for R'. In that study, antisera were prepared in guinea pig by injecting purified bovine heart muscle type II protein kinase. They demonstrated that both the type II holoenzyme and R' competed in an identical manner for [3H]-R' or [3H]-protein kinase holoenzyme. However, C and partially purified type I protein kinases eluted from DEAE-cellulose did not cross-react in the immunoassay. This observation suggested that the type II holoenzyme antiserum was only recognizing determinants on the R', but not on C. They further demonstrated that intertissue and interspecies similarities and differences exist in the structure of these protein kinases. Rubin et al. (14) extended these studies to soluble and particulate fractions of bovine cerebrum cortex by utilizing type II protein kinase immunoassay and antibody affinity chromatography, reporting that the bovine cerebral cortex CAMP-dependent protein kinases possess many of the characteristics of bovine heart soluble type II protein kinase and possess antigenic determinants that are related to, but readily distinguished from, determinants of type II heart soluble protein kinase.

The present study was undertaken in order to probe by immunological techniques the structure, function, and mechanism of action of cAMP on the type I cAMP-dependent protein kinase. The production of specific R' antisera has allowed us to develop radioimmunoassay for R'. As a part of this work, a sensitive and specific radioimmunoassay of bovine skeletal muscle R' was developed. This paper describes the characteristics of the radioimmunoassay in utilizing several different antisera to R'. In addition, the effect of cAMP and bovine skeletal muscle C on the immunoreactivity of bovine skeletal muscle R' are presented in this communication. A preliminary report of some of these investigations has appeared in abstract form (18).
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MATERIALS AND METHODS

Chemicals—ATP, CAMP, GMP, histone type IIa, ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), EDTA, Mes, bovine albumin, chloramine T, sodium dodecyl sulfate (SDS), sodium metabisulfite, anti-rabbit immunoglobulin G (goat), Freund’s complete and incomplete adjuvant, carrier-free Na125I 
(13 to 17 mCi/μg) in 0.1 M NaOH (pH 7 to 10), and DEAE-cellulose (Whatman DE52) were obtained from commercial sources. Immediately before use, 8 M urea solution was passed over a column of mixed bed ion exchange resin. Electrophoresis and chromatography were obtained from Bio-Rad Laboratories. Sephadex G-25M, PD-10 column, Sephadex CM-50, and Sephacryl S-200 were obtained from Pharmacia Fine Chemicals.

Purification of Protein Kinases and Their Subunits—Bovine skeletal muscle type I and bovine heart muscle type II protein kinases were partially purified on DEAE-cellulose as described previously (19). The H-CAMP complex of bovine skeletal muscle and bovine heart muscle protein kinases were prepared by utilizing DEAE fractionation and immobilized N'-C-CAMP affinity chromatography as described previously (20, 21). R1° often required further purification on Sephacyr S-200 to remove trace contaminants and was isolated as a mixture of the phospho and dephospho forms. R1° was prepared by incubations in the presence of substrates and R1° was then molar excess of C in high speed in a 10 mM Mes buffer (pH 6.5), 15 mM β-mercaptoethanol, 0.1 mM EDTA containing 0.1 mM ATP, and 1 mM MgCl2. Excess free C was then removed by passage over a Sephadex CM-50 column. The recombined enzyme sedimented as a single species with a x20 value of approximately 70, as described by Schwechheimer and Hofmann (22). Bovine skeletal muscle C was prepared utilizing a “tricolumn” modification of previously published procedures (17, 19). Bovine lung G-kinase was prepared utilizing 8-12 aminoethylamino-CAMP Sepharose affinity chromatography and gel filtration on Sephacyr S-200 as described (21, 23). Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate was conducted by the method of Weber and Osbourn (24), except that a 25 mM Tris, 120 mM glycine buffer system (pH 8.5) was used in the place of sodium phosphate buffer. Calculation of molar concentrations of protein kinases used in these experiments are based upon the molecular weight of 170,000 for R1°, 90,000 for R1°, 110,000 for R1°, 40,000 for C, and 160,000 for G-kinase.

Preparation of Cyclic Nucleotide-free R1° by Urea Treatment—Cyclic nucleotide-free R1° and R1° were prepared as described by Builder et al. (25). R1° was prepared by this procedure exhibited identical properties to histone type IIa as a substrate (19). One unit of activity is defined as that amount of enzyme catalyzing the transfer of 1 nmol of phosphate to histone type IIa per min. Protein concentrations were determined either by the method of Lowry et al. (29) or that of Bradford (30) with bovine albumin as standard.

RESULTS

Characterization of R1° Antisera—Specific precipitating antisera to bovine skeletal muscle R1° were raised in five rabbits by repeated immunization with homogeneously pure preparations of R1°. Of the animals tested, all animals immunized

small conical plastic tube. The reaction was allowed to proceed at room temperature for 90 s and was terminated by the addition of 10 mM Mes buffer, pH 6.5, 15 mM β-mercaptoethanol, 0.1 mM EDTA, 50 mM NaCl. 125I-R1° was isolated from unreacted Na125I by applying the reaction mixture to a Sephadex G-25 column (Pharmacia PD-10 column) equilibrated with 10 mM Mes buffer, pH 6.5, containing 15 mM β-mercaptoethanol, 0.1 mM EDTA, 50 mM NaCl, and 0.25% (w/v) bovine albumin. A specific activity of 18,000 to 80,000 dpm/ng was obtained and about 97% of the radioactivity recovered from the exclusion fraction of the G-25 column was found to be precipitated with 10% trichloroacetic acid. The immunoreactivity of the tracer was found to be stable for 3 months.

Radioimmunoassay of R1°—All immunosassays were carried out in 10 mM Mes buffer, pH 6.5, 15 mM β-mercaptoethanol, 0.1 mM EDTA, 50 mM NaCl, 0.25% bovine albumin. Several different conditions were tried for the radioimmunoassay, and the best of these conditions are given here. All incubations were performed in duplicate or triplicate in nonalkoxylated polystyrene tubes (10 × 75 mm) (Falcon Plastics). Each test tube contained (in order of addition) 100 μl of R1° standard or unknown solution containing kinases or other subunits, 100 μl of appropriately diluted antiserum in 1% normal rabbit serum (final dilution 1.000 to 1.10,000), and 100 μl of 125I-R1° (10,000 to 20,000 cpm) in a final volume of 300 μl. These assays were set up at 4°C and primary incubations were at 4°C for 24 h. This was followed by the addition of 200 μl of goat anti-rabbit immunoglobulin G antiserum which had been previously diluted so as to give maximum precipitation of the complexes (28). The tubes were incubated at 4°C for 16 h and then centrifuged at 4°C for 15 min at 3,000 × g. The supernatants were discarded by aspiration and residues were washed twice with 2 ml of buffer and counted in a gamma counter. The dilution of antiserum was found to be sufficient to bind 20 to 90% R1°. The percentage of bound radioactivity was calculated as a function of total radioactivity by dividing the radioactivity precipitated with antibody minus radioactivity precipitated with nonimmune serum by total radioactivity added to the incubation.

Miscellaneous—Phosphotransferase activity was determined by using mixed histone type IIa as a substrate (19). One unit of activity is defined as that amount of enzyme catalyzing the transfer of 1 nmol of phosphate to histone type IIa per min. Protein concentrations were determined either by the method of Lowry et al. (29) or that of Bradford (30) with bovine albumin as standard.

Fig. 1. Direct binding curve. Various amounts of rabbit anti-R1° antisera were mixed with rabbit normal serum. Each tube contained an identical 90 μl of total rabbit serum and was incubated with 125I-R1°. Binding of 125I-R1° was determined in each assay tube as described under “Materials and Methods.”
with R' produced antisera in a titer (>40% \(^{125}\text{I}-\text{R}_{\text{II}}\) binding) from 500 to 10,000 after multiple immunizations.

**Standardization of Radioimmunoassay**—Several different parameters were checked for optimum binding in the immunoassay such as effect of pH, bovine albumin concentration, and time for equilibration of the antigen-antibody reaction. A concentration of 0.25% bovine albumin was found to be adequate as the protective agent for the antigen-antibody equilibrium. The equilibration of antigen-antibody was completed in 21 h at 4°C. Maximal binding in the assay was obtained in 10 mM Mes, 0.1 mM EDTA, 15 mM \(\beta\)-mercaptoethanol (0.1%), 50 mM NaCl, 0.25% bovine albumin, pH 6.5. It had been shown earlier that \(\beta\)-mercaptoethanol and EDTA are known to prevent the degradation of labeled antigens during immunoassays of polypeptide hormones (28) and are used for the purification and stabilization of cyclic nucleotide-dependent protein kinases and their subunits (17, 21). In the R' immunooassay system, the binding increased progressively when the EDTA and \(\beta\)-mercaptoethanol were included in the incubation mixture. Moreover, inclusion of these agents facilitated the estimation of R' and type I protein kinase concentrations in the various tissue extracts and partially purified preparations of protein kinases from DEAE-cellulose.

**Direct Binding and Standard Curves**—After optimizing conditions, direct antiserum binding experiments were carried out using different volumes of the R' antiserum as described under “Materials and Methods.” Fig. 1 depicts an example of the curve obtained with R' antiserum. Up to 80% of the \(^{125}\text{I}-\text{R}_{\text{II}}\) was bound in the double antibody method described here. It is probable that even more total binding of \(^{125}\text{I}-\text{R}_{\text{II}}\) could have been precipitated had more antiserum been employed, since it is evident that plateaus had not been reached at the 2-\(\mu\)l point. In all experiments, the background radioactivity precipitated by nonimmune serum was 5 to 8% of the total cpm added.

Fig. 2 depicts a competition assay using different amounts of unlabeled \(\text{R}_{\text{II}}\). Beef skeletal muscle \(\text{R}_{\text{II}}\) competed (0.003 cpm added. It is probable that even more total binding of \(^{125}\text{I}-\text{R}_{\text{II}}\) could have been precipitated had more antiserum been employed, since it is evident that plateaus had not been reached at the 2-\(\mu\)l point. In all experiments, the background radioactivity precipitated by nonimmune serum was 5 to 8% of the total cpm added.

**Specificity of Antiserum in the Immunoassay**—In order to quantify the relative specificity of binding, the effects of various cyclic nucleotide-dependent protein kinases and their subunits were investigated on the \(^{125}\text{I}-\text{R}_{\text{II}}\) binding to R' antiserum in the immunoassay (Fig. 9). C and \(\text{R}_{\text{II}}\), up to 10,000-fold greater concentrations, did not compete with \(^{125}\text{I}-\text{R}_{\text{II}}\) binding. At least 100 pmol of C and \(\text{R}_{\text{II}}\) were required to produce apparent 0.0025 and 0.030% minimal competitive cross-reactivity in the immunoassay. Homogeneously pure preparations of G-kinase up to 1.0 pmol did not affect the binding of \(^{125}\text{I}-\text{R}_{\text{II}}\) to R' antiserum in the immunoassay. At least 100 pmol of this enzyme was required to produce 0.13% competitive cross-reactivity in the immunoassay.

**Sensitivity of R' Antisera in the Immunoassay**—The detection limit of the immunoassay is governed by the energy of the antigen-antibody reaction. Thus, the detection limit using the same specific activity of radiolabeled antigen varied with the antiserum employed. The sensitivity of various R' antisera is shown in Table I. The antisera showed different degrees of sensitivity in the immunoassay. The sensitivity of 3R' \(\text{R}_{\text{II}}\), and 3R' No. 3 antisera were 10- to 30-fold higher than the \(\text{R}_{\text{II}}\) immunoassay (13).

**Radioimmunoassay of Type I Protein Kinase in Bovine Tissue**—The quantitation of R' in various preparations was determined by measuring the displacement of \(^{125}\text{I}-\text{R}_{\text{II}}\) either by varying amounts of \(\text{R}_{\text{II}}\) or \(\text{R}_{\text{III}}\). Only those sera which exhibited similar degrees of inhibition of R' antisem binding of \(^{125}\text{I}-\text{R}_{\text{II}}\) by increasing concentrations of \(\text{R}_{\text{II}}\) and \(\text{R}_{\text{III}}\) were used in the immunoassay (Fig. 3). The concentration of \(\text{R}_{\text{II}}\) in the immunoassay is plotted as protein kinase catalytic activity (units/ml) assessed in the presence of cAMP. The

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**Sensitivity of R' Antisera in the Immunoassay**—The detection limit of the immunoassay is governed by the energy of the antigen-antibody reaction. Thus, the detection limit using the same specific activity of radiolabeled antigen varied with the antiserum employed. The sensitivity of various R' antisera is shown in Table I. The antisera showed different degrees of sensitivity in the immunoassay. The sensitivity of 3R', 4R', and 3R' No. 3 antisera were 10- to 30-fold higher than the R' immunoassay (13).
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catalytic activity of bovine skeletal muscle R2'C2 was compared with the other tissue extract catalytic activity.

Fig. 4 depicts the competitive cross-reactivity of cAMP-dependent protein kinase activity of various bovine tissue extracts in the immunoassay. Extracts of each of the tissues assayed showed parallel competition to that produced by R2'C2. However, the ability of 1 unit of protein kinase activity to displace 125I-R1U binding varied from one tissue extract to another. The apparent C activity of immunoassayable protein kinase was higher for bovine kidney, liver, heart, and brain than for pure or crude skeletal muscle. These differences appear mainly due to the presence of different concentrations of type I and type II cAMP-dependent protein in various tissues (5). This observation is in agreement with the data on

![Fig. 4. Comparison of inhibition curve produced by 12,000 x g supernatant fluid of bovine tissue extracts to that of pure bovine skeletal muscle R2'C2. Binding of 125I-R1U was determined in each tube as described under "Materials and Methods." Points are the mean of duplicate incubation. A, pure R2'C2; B, skeletal muscle; C, kidney; D, liver; E, heart; F, brain.](http://www.jbc.org/)

![Fig. 5. Comparison of inhibition curve produced by 12,000 x g supernatant fluids of rat tissue extracts to that of pure bovine skeletal muscle R2'C2. Binding of 125I-R1U was determined in each tube as described under "Materials and Methods." Points are the mean of duplicate incubation. A, pure R2'C2; B, muscle; C, testis; D, liver; E, heart; F, brain.](http://www.jbc.org/)

Fig. 6. Effect of cAMP on the inhibition of site-specific 3R' No. 3 antisem binding of 125I-R1U by increasing concentrations of R1U. CAMP (10 µM) was incubated with various concentrations of R1U (0.1 to 100 pmol) and 125I-R1U and binding was determined as described under "Materials and Methods." Note that cAMP had specific effect on the immunoreactivity of R1U, but not on the labeled 125I-R1U.

R1 immunoassay, where catalytic activity of immunoassayable protein kinase of various tissues was greater than pure bovine heart protein kinase (13).

In addition, the antisem was utilized to measure the immunoreactivity of R1 during bovine skeletal muscle type I protein kinase purification. When crude tissue extracts, partially purified Peak I enzyme from DEAE cellulose, and R2'C2 were tested, partially purified preparations produced immunocross-reactivity in a parallel fashion to pure R2'C2 and in direct proportion to their enzymatic activity. This suggests that a close correlation exists between the ratio of phosphotransferase activity and immunocross-reactivity of R1. However, beef skeletal muscle crude extract did not contain all immunoassayable type I cAMP-dependent protein kinase. Approximately 20 to 30% of the protein kinase eluted with 10 to 20 mmoj conductivity (19) did not cross-react significantly in the R1 immunoassay. Similar results were obtained with partially purified Peak II bovine heart muscle enzyme (data not shown).

Species Specificity of Radioimmunoassay—The curves shown in Fig. 5 depict the competitive cross-reactivity of various rat tissue extracts in the immunoassay. The cAMP-dependent protein kinase activity in these preparations exhibited up to 20% inhibition of R1 antisem binding of 125I-R1U. The concentration response of various rat tissue extracts was not parallel to that produced by beef skeletal muscle R2'C2. Moreover, the ability of tissue phosphotransferase activity to inhibit 125I-R1U binding in the immunoassay varied from one tissue extract to another. These findings are in agreement with those of the R1 immunoassay (13). Because of this lack of parallelism, an accurate estimate of the concentration of immunoassayable protein kinase could not be made.

Specific Effects of CAMP and Bovine Skeletal Muscle C on the Immunoreactivity of R1—Among the five antisera screened, one antisem detected site-specific effects of CAMP and C on the immunoreactivity of R1 in the immunoassay. Addition of CAMP to R1U produced a 3- to 10-fold decrease in inhibition of 125I-R1U binding by increasing concentrations of R1U (Fig. 6). A significant decrease in the immunoreactivity of

5 When rat skeletal muscle and rat heart muscle extracts were treated using a combination of antisera specific to R1 and bovine heart R1 followed by double antibody precipitation, it was found that 4- and 1.2-fold more cyclic AMP-dependent protein kinase activity was precipitable than with R1 antisem alone.
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The immobilization of a sensitive and accurate immunoassay for the R' depends partly upon the production of high affinity antibodies of desired specificity can be screened for immunocross-reactivity between species and between specific tissues in the same animal species. Moreover, a similar point was also observed for the cross-reactivity of DEAE-cellulose-fractionated rabbit Peak I and Peak II enzyme with antisera specific to R' and R". A similar point was also observed for the cross-reactivity of various bovine and rat tissues. Although protein kinases prepared from crude rat tissue extracts exhibited nonparallel cross-reactivity in the R' immunoassay, they were precipitated by the combined action of CAMP and C. A similar point was also observed for the cross-reactivity of DEAE-cellulose-fractionated rabbit Peak I and Peak II enzyme with antisera specific to R' and R". Moreover, a similar and specific immunocytochemical localization of R' in various bovine and rat tissues supports the findings of immunocross-reactivity between species and between specific tissues in the same animal species.

In general, R' antisera obtained from several animals did not differentiate immunoreactivity of R', R'CAMP, and R'C2 in the immunoassay. This suggests that the nature of association between R' and CAMP or R'C2 and C allow identical recognition of free R' subunit by most of the R' antisera. However, a site-specific antiserum (3'R' No. 3) was screened which differentiated immunoreactivity of free R', R'CAMP, or R'C2 in the immunoassay. These findings suggest that specific binding of cAMP and C to R' causes blocking of antigenic determinants and/or changes in the conformation of the molecule. The presence of such site-specific antibodies has been described in other systems (32). Moreover, these findings will provide an experimental framework from which antibodies of desired specificity can be screened for immunocytochemical localization of R' in various tissues.

In future experiments, the availability of such an immunoassay should facilitate studies designed to probe the antigenic determinants of R' in various tissues.
determinant(s) of proteolytic fragments of R' and R'\textsuperscript{II} (8, 33). The antibodies purified for each fragment, for example, can be utilized as a molecular probe for studying the refolding (34) and determining the multiple different domain-specific antigenic site(s) of R' (35). Although this immunoassay is more sensitive than enzyme assay and detects concentrations of R' or R'\textsubscript{II} in crude extracts or partially purified preparation of bovine skeletal muscle, it cannot be utilized to measure the concentrations of R' in other tissues in the same animal species and between species. In the future, the production of specific antibody to R' by the hybridoma monoclonal technique (36) might generate antisera which recognize a specific antigenic determinant that has the same degree of cross-reactivity toward R' between species and between various tissues in the same animal species.

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