Vol. 99, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 31, 1981 Pages 475-483

QUANTITATION OF HETEROLOGOUS PROTEIN KINASE SUBUNITS R I AND R II WITH THE AID OF TYPE SPECIFIC ANTIBODIES

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Received February 10,1981

SUMMARY

Specific antibodies to rabbit type I and bovine type II regulatory subunits R of cAMP dependent protein kinases were used for a procedure that allows quantitation of the R I and R II isoproteins. It is based on the sedimentation of the antigen-antibody complexes in the presence of Protein A-Sepharose. Under these conditions absolute amounts of homologous and heterologous R I and R II proteins could be determined in extracts by immunotitration. Multiple forms of R I and R II could also be discriminated: After photoaffinity-labeling with (^{32}P)azido cAMP, the isoproteins were specifically sedimented by the corresponding antiserum in the presence of Protein A-Sepharose, and the SDS eluates of the sediments were analyzed by gel electrophoresis and autoradiography.

INTRODUCTION

In most mammalian tissues, two types of cAMP-dependent protein kinase (holoenzymes $R_2 \cdot C_2$) are found, which differ in their regulatory subunits (R I, R II), the catalytic subunit C apparently being identical (1, 2). Little is known about the significance of these isoenzymes which have been implicated in the regulation of cell proliferation, differentiation and hormonal stimuli (3-5). Further, recent evidence suggests functions of R proteins that are independent of C activity (6-9) or may involve translocation processes (cf. 10-13). Analysis of the isoenzymes, however, is difficult. DEAE cellulose chromatography usually performed is prone to erroneous results especially in cases of partial activation of protein kinases (cf. 10). Identification of R I and R II proteins by affinity labeling (14) is based on the determination of molecular weights and therefore not suitable to tissue extracts containing unknown forms. Determinations by radioimmunoassay (cf. 15, 16) of absolute amounts of antigenic proteins is only possible in homologous tissues, or in tissues from which the pure proteins have been isolated for standardization of the test. In this study we describe a procedure which overcomes these difficulties. It uses type-specific antibodies in combination with Protein A-Sepharose to separate R isoproteins and to classify multiple forms of R I and R II.

0006-291X/81/060475-09$01.00/0

MATERIALS AND METHODS

Antigens: Regulatory subunits R of cAMP dependent protein kinase were prepared from rabbit skeletal muscle (type I) and from bovine heart muscle (type II) as previously described (17). Both antigens were homogeneous as shown by SDS gel electrophoresis.

Antibodies: Anti - R I was raised in guinea pigs by the injection (i.m.) of 200 µg R I followed by two booster injections (s.c.) of 70-100 µg R I in intervals of 4 weeks. The animals were killed and bled one week after the last booster. Anti - R II was raised in female rabbits by subcutaneous injections of 500 µg R II, using complete Freund's adjuvant. Three booster injections (s.c.) with 200 µg R II followed at 4 weeks intervals. The animals were bled through the ear veins 12 days after the last booster injection. For use in the Ouchterlony test, y-globulins were separated by an ammonium sulfate fractionation. Pure anti - R I y-globulins were prepared by affinity chromatography using R I immobilized by reaction with CNBr-activated Sepharose 4B. The antibodies retained on the column were eluted with 6 M urea and renaturated by dialysis.

Immunotitration of R proteins in the presence of Protein A-Sepharose: Samples (1 pmol cAMP binding sites) were incubated for 2 hrs at 4°C in a total volume of 150 µl containing 25 mM phosphate buffer pH 7.2, 5 mM EDTA, 2 mM dithiothreitol, 50 mM NaCl, 0.5 mM methylisobutylxanthine (MIX), 0.02% NaN_3, 500 µg protein (serum albumin or antiserum protein), 10 µl (settled gel) Protein A-Sepharose CL 4B (Pharmacia), and 0.1 µM (2,8-^{3}H)cAMP (8Ci/mMol; Amersham-Buchler). During incubation, the tubes were rotated. When (pure) R proteins containing bound cAMP were analyzed, the samples were pre-incubated at 37°C for 30 min to facilitate (^{3}H)cAMP exchange. Following incubation, excess cAMP was removed by the addition of 80 µl charcoal suspension (100 mg/ml buffer pH 7.2 containing 25 mM phosphate, 0.5 mM EDTA, 0.5 mM MIX, 0.05% NaN_3 and 20 mg serum albumin). After thorough mixing, the suspension was centrifuged (10 000 g/4 min, Eppendorf minifuge), thus sedimenting charcoal and Protein A-Sepharose-bound antigen/antibody complex. (^{3}H)cAMP-labeled non-neutralized R proteins remaining in the supernatant were quantified by liquid scintillation counting.

Photoaffinity labeling: cAMP binding proteins were labeled by photoactivated reaction with (^{32}P)8-azido cAMP (18): Tissue extracts containing 1-2 pmol cAMP binding capacity, 20 mM Tris·HCl pH 7.4, 20 mM benzamidine and 4 mM EDTA were incubated with 0.9 µM (^{32}P)azido cAMP (ICN, 10-80 Ci/mMol) for 1 h at 4°C in a total volume of 50 µl. The samples were exposed to UV light from a Hanau NN 15/11 lamp for 10 min at a distance of 4 cm. The reaction mixture was analyzed by SDS polyacrylamide gel electrophoresis (cf. 19). Kodak XOmat films were exposed to the dried gels at -80°C.

Immunological discrimination of R I and R II variants: Photoaffinity labeled extracts prepared as described above were incubated with antisera (amounts corresponding to the endpoints of titration) in the presence of 20 µl Protein A - Sepharose for 2 hrs at 4°C. After centrifugation the gel was washed 3 times with 150 mM NaCl - 5 mM tris pH 7.4, and once with the 'labeling buffer'. The antigens were released into solution by treatment with SDS (3%) - electrophoresis sample buffer and analyzed together with the non-separated R proteins in the extracts.

RESULTS

1. Preparation of antibodies

The presence in the rabbit sera of anti-R II antibodies could be demonstrated with the Ouchterlony double diffusion technique. Anti-R I antibodies produced in guinea pigs proved to be non-precipitating. Their presence was demonstrated

by comparative electrophoresis of anti R I globulin (purified by affinity chromatography) and its complex with the R I antigen (not shown).

2. Immunotitration of R proteins with the aid of Protein A-Sepharose

In order to achieve a wide applicability of the antibodies to the quantitation of R proteins, we decided to use immunotitration. cAMP receptor proteins were saturated with (^{3}H)cAMP and incubated with increasing amounts of antiserum. Charcoal was then added to remove excess (^{3}H)cAMP. A single centrifugation step sedimented charcoal and the insoluble antigen - antibody complexes, R-(^{3}H)cAMP in the supernatant serving as titration indicator. This procedure requires precipitating antibodies which, however, were not present in the anti-R I antisera. Since it is known that the staphylococcal Protein A is able to form tight complexes with the fc section of IgG antibodies (cf. 20), Sepharose-bound Protein A was included in the titration mixture. Under these conditions, complete sedimentation of R I could be achieved (Fig. 1A). Experiments with control serum demonstrated the selectivity of the procedure (not shown). Even in the case of anti-R II antisera (Fig. 1B), the use of Protein A-Sepharose much improved the precipitating capacity of the antiserum.

3. Type-specificity of antibodies

Protein A-Sepharose-aided immunotitration was applied to analyze the specificity of both immune sera. When 60 ng R I protein was titrated with anti-R I, 0.16 µl antiserum were required for 50% neutralization (Fig. 1C), whereas no sedimentation was seen with R II. Titration of the type II protein with anti-R II (50% neutralization at 0.017 µl antiserum) again proved to be specific, no cross-reaction occurring with the R I isoprotein (Fig. 1D). As the regulatory subunits of protein kinases are known to be subject to limited proteolysis (cf. 10), degradation products were also analyzed: R I' and R II' products (M_r 37000), obtained from the native R proteins by trypsin treatment (21) still retained the antigenicity, although interaction with the antibodies was considerably weaker, requiring 10 to 30 times higher concentrations of antiserum for 50% neutralization.

We also determined the undissociated holoenzymes of protein kinases (DEAE cellulose fractions from rabbit muscle (PK I), and from bovine heart (PK II), respectively, (^{3}H)cAMP in this case being added after separation of the antigen-antibody complexes. Surprisingly, titration curves did not differ from those

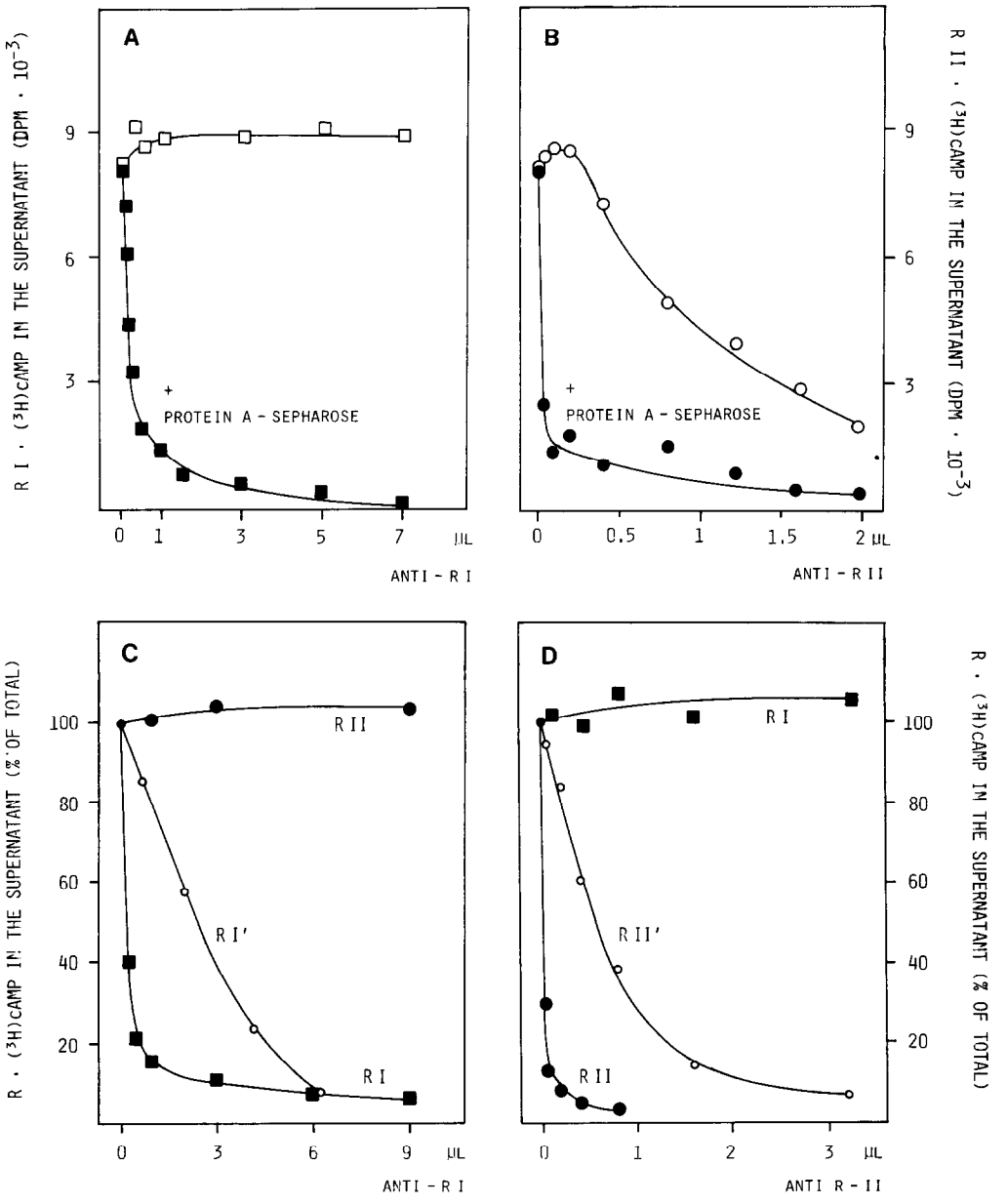

Figure 1 Immunotitration of R proteins in the presence of Protein A-Sepharose
Regulatory subunits R I (A) and R II (B) were titrated with their respective antisera in the presence and absence of 10 µl Protein A-Sepharose gel as described in Methods. The reactivities of anti-R I and anti R-II towards the R subunits and their products of limited proteolysis R' are demonstrated in (C) and (D).

with free regulatory subunits. The amounts of antisera required to produce 50% precipitation of (³H)cAMP binding activity were essentially the same as in the case of the pure R proteins. The test for free catalytic subunits indicated that

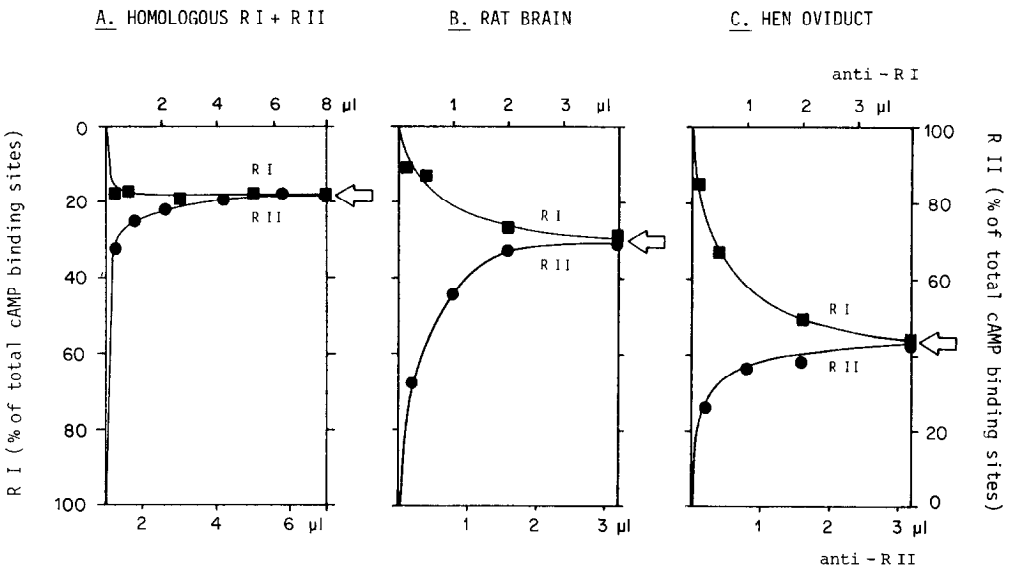

Figure 2 Determination of R I / R II ratios by immunotitration

Samples containing both types of R proteins were titrated with anti - R I (upper curve) and anti - R II (inverse lower curve). In this way, the endpoints of both titrations are marked by the intercept on the scale at the right (arrow). Total cAMP binding sites measured in the supernatant are set 100%. (A) Mixture of purified antigens (R II: 1.6 pmol; R I: 0.4 pmol cAMP binding sites); (B) rat brain extract; (C) hen oviduct extract. Tissues were pulverized under liquid nitrogen (Dismembrator; Braun, Melsungen), followed by homogenization with 6 vol of buffer pH 7.4 containing 20 mM Tris·HCl, 20 mM benzamidine, 4 mM EDTA, 15 mM mercaptoethanol and 100 E / ml Trasylol (Bayer, Leverkusen), and centrifugation (5 min 10 000 g).

the reaction of the holoenzymes with the antibodies did not lead to significant dissociation (not shown). The two antisera, therefore, allowed detection and quantitation of each R protein whether present as free regulatory subunit or in the form of the holoenzyme. From these and the preceding data it is clear that both, anti-R I and anti-R II antisera were type-specific. A prerequisite for the determination of the R isoproteins in the presence of each other was thus fulfilled.

4. Determination of R I and R II in homologous and heterologous tissues

When a mixture of homologous R I and R II was subjected to the immunotitration with anti-R I, a proportion of the total cAMP binding sites (= total R) was sedimented, that corresponded to the amount added. The "end-point" is marked by the plateau value finally reached (Fig. 2A). Titration with anti-R II precipitated the residual part of R protein. This procedure is not only applicable to homologous R proteins. Proteins from heterologous tissues can also be quantitated provided they exhibit reactivity with the anti-rabbit R I and anti-bovine R II

antibodies, respectively. In the case of lower cross-reactivity, higher amounts of antisera are required to neutralize all of the R antigen, i.e. to reach plateau values. From the absolute value of total R (total high affinity cAMP binding sites) and the proportions of R I or R II determined by immunotitration, absolute values of type I and type II proteins can be calculated.

When extracts from rat brain were titrated with the two antisera in the presence of Protein A-Sepharose, proportions of total cAMP binding sites were neutralized that corresponded to 30% type I and 70% type II R proteins (Fig. 2B). The procedure was also applied to the quantitation of R proteins in tissue extracts from other organs and other species. The following R I/R II ratios were found:

rat liver (55/45); rat heart (75/25); mouse Ehrlich ascites tumor (65/35); rabbit muscle (75/25); bovine kidney (15/85); human kidney (38/62); chronic lymphocytic leucemia lymphocytes (63/37).

The cross-reactivity of R proteins even from an avian tissue (hen oviduct) was sufficiently high to allow titration of the R proteins (R I/R II = 57/43; Fig. 2C).

5. Classification of multiple R forms as type I and type II isoproteins

Photoaffinity labeling with (^{32}P)azido cAMP of various tissue extracts in the presence of protease inhibitors followed by electrophoresis and autoradiography had demonstrated the existence of multiple forms of R proteins (cf. 10). Inclusion of a separation step with Protein A-Sepharose-bound antibodies allowed to isolate and to classify the individual cAMP-receptors as type I (type II) R isoproteins. A corresponding analysis of hen oviduct using the anti-rabbit R I and the anti-bovine R II is shown in Fig. 3 demonstrating the presence of an R I 49 000, and R II 54 000, and eliminating the other labeled components as unspecific cAMP binders (also shown by the use of excess cold cAMP). Another example concerns extracts of human kidney cortex (pericarcinomatous) which exhibit a broad band near 49 000 molecular weight. Upon treatment with anti-R I and anti-R II, it resolves into two distinct components: the common R I (49 000) and a cAMP receptor protein with an apparent molecular weight of 50 000. Because of the reactivity towards anti-R II, this protein is to be classified as a new type II isoprotein.

DISCUSSION

The data presented in this study show that the regulatory subunits R I and R II are immunologically distinct proteins, confirming previous reports (15, 16). Consequently, the use of specific anti-R I and anti-R II antibodies in combination with (^{3}H)cAMP binding and Protein A-Sepharose allowed the quantitation of R I and R II isoproteins from various sources by immunotitration. Though type-specific, the antisera lack a pronounced species specificity which may be the

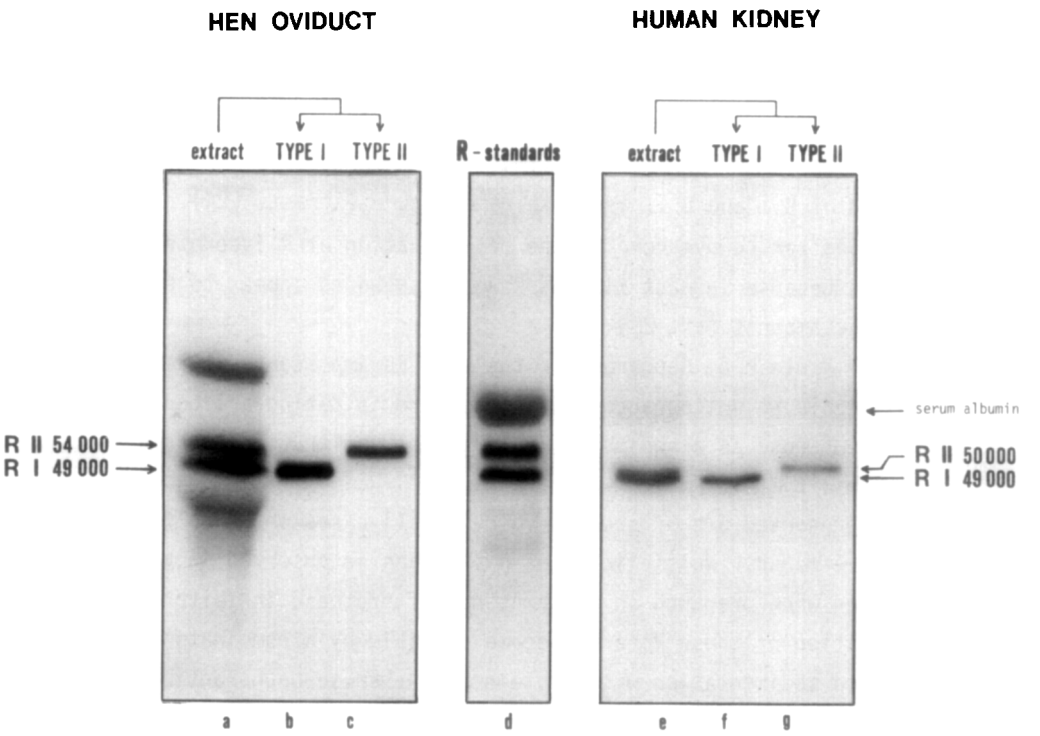

Figure 3 Autoradiography of affinity-labeled R proteins in extracts and in sedimented immune complexes

Extracts (cf. fig. 2) were affinity-labeled with (^{32}P)azido cAMP. Aliquots were treated with antisera in the presence of Protein A-Sepharose. Extracts and sedimented antigen-antibody complexes were subjected to autoradiography after SDS gel electrophoresis (see Methods). (a) hen oviduct extract; (b) and (c) anti-R I and anti-R II reactive components of hen oviduct; (d) R I (0.03 µg) and R II (0.06 µg) references affinity-labeled in the presence of bovine serum albumin (30 µg); (e) human kidney cortex removed by surgery of a hypernephroma, apparently normal tissue; (f) anti-R I reactive and (g) anti-R II reactive components of kidney extracts.

result of relatively conserved structures of the R proteins. This becomes an advantage when the antibodies are applied to the quantitation of heterologous R proteins. Our procedure allowed to determine R isoproteins from all mammalian species so far tested, and from an avian organism.

It was observed that dissociated and undissociated holoenzymes require the same amounts of antisera. A corresponding observation has been made with anti-C antisera (23). This suggests that the domains involved in R : C binding are conserved non-antigenic structures (cf. 22).

There is some evidence that the relative concentrations of protein kinase I and II may account for a certain specificity of cAMP-mediated stimuli (cf.3-5). Analysis of PK I/PK II ratios usually performed by DEAE cellulose chromatography,

however, may be subject to errors in the case of partial activation, or when limited proteolysis produces forms of the two protein kinase isoenzymes that lead to multiple undefined peaks (10). Also, photoaffinity labeling, though useful for the detection of the cAMP receptor proteins, was shown to be of limited value for the quantitation of R I and R II isoproteins in crude extracts (24).

These difficulties can be overcome by the determination of R isoproteins by immunotitration, because in most tissues R and C subunits appear to be present in stoichiometric amounts (cf. 25).

The inclusion of Protein A - Sepharose in the test is important. It renders the procedure applicable to antisera containing non-precipitating antibodies. Use of Protein A - Sepharose as a sedimenting aid in immunotitration appears to have general applicability (cf. 23).

The procedures presented are of special value in all cases where multiple forms of R proteins are found. Multiplicity of R proteins is observed in a variety of extracts, even when prepared in the presence of protease inhibitors (cf. 22, 26). Classification of these R forms became possible by a combination of affinity labeling and sedimentation with Protein A - Sepharose-bound antibodies. In this way, a new variant (M_r 50 000) was found in extracts from several human tissues and identified as a type II isoprotein. In addition, several forms with molecular weights in the range of 35 000 - 40 000, presumably derived from the native subunits by limited proteolysis, could be classified.

ACKNOWLEDGEMENTS

This study contains parts of the doctoral thesis of H.S. (Universität Hamburg, Fachbereich Biologie). We would like to thank Drs. H. Lang and S. Neumann (Biochem. Forschung, E. Merck, Darmstadt) for their help in raising antibodies to rabbit R I. The work was supported by the Deutsche Forschungsgemeinschaft, SFB 34.

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