PURIFICATION OF THE REGULATORY SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE BY MONOCLONAL ANTIBODIES

I. R. Éshba, N. A. Kochanova, K. M. Popov,
T. V. Bulargina, and E. S. Severin

UDC 616.008.931:616-008.93:
577.123.3]-078.73

KEY WORDS: monoclonal antibodies; regulatory subunit of protein kinase; cAMP; immunoaffinity sorption

cAMP-dependent protein kinase (PK) exists as two isozymes in most mammalian tissues. The holoenzyme consists of two types of subunits: regulatory (R), which binds cAMP, and catalytic (C), which is responsible for the phosphotransferase reaction [5, 8, 11]. The two types of holoenzymes are distinguished by elution from DEAE-cellulose and are designated type I and type II [1, 7]. Differences are due to the structure of R.

Whereas the C subunit can be isolated in a homogeneous state by the use of traditional methods, isolation of R in the homogeneous state became possible only by the use of affinity chromatography. However, the use of this latter method, which shortens the time of isolation of R considerably, still requires at least 5 days to purify RII. Shortening this isolation time is particularly important because of the rapid endogenous proteolysis of RII.

The most suitable method of shortening the isolation time in order to obtain intact RII could be the immunoaffinity method.

This paper describes the results of isolation of RII in the homogeneous state with the aid of an immunoaffinity sorbent based on monoclonal antibodies (MCA) to RII.

EXPERIMENTAL METHOD

The RII subunit of cAMP-dependent PK was isolated for immunization by methods [2, 9] with the following modification: elution with DEAE-Sephadex A50 was done in stages with buffer containing 10 mM Tris, 2 mM EDTA, and 200 and 400 mM NaCl.

cAMP-binding activity was determined by the method in [3]. Sp 2/0 mouse myeloma cells were grown in Eagle's medium in Dulbecco's modification (DMEM) with 10% fetal calf serum, 10% normal horse serum, 5% sodium bicarbonate, 1% sodium pyruvate, 2% glutamine, and 0.1% gentamicin (pH 7.2).

BALB/c mice were immunized intraperitoneally twice, with 100 μg of RII each time, with an interval of 3-4 weeks, followed by hyperimmunization with 50 μg, intravenously. The mice were killed 3 days after hyperimmunization by cervical dislocation and the spleen was removed to obtain splenocytes. The splenocytes were fused with myeloma cells by the method in [4]. The specificity of the resulting clones was determined by ELISA, using a conjugate of rabbit antimouse antibodies with peroxidase. The substrate for peroxidase was 2.2-azino-bis-(3-ethyl-benzothiazoline-6-sulfonic acid (ABTS).

MCA were purified on columns with protein A-sepharose. The type of MCA was determined by the double diffusion method [6]. Electrophoresis was carried out by Laemmli's method with 6%

TABLE 1. Characteristics of Monoclonal Antibodies

Antigen	Clone	Titer in ascites fluid	Protein, mg/ml	Class, subclass
R_2II	1E6	50 000—200 000	5	M
	2A10	400 000—6 500 000	6	G_1
	3E9	100 000—400 000	3	G_1

Biological Faculty, Moscow State University, Moscow. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 103, No. 5, pp. 567-569, May, 1987. Original article submitted July 3, 1986.

0007-4888/87/0005- 0643 $12.50

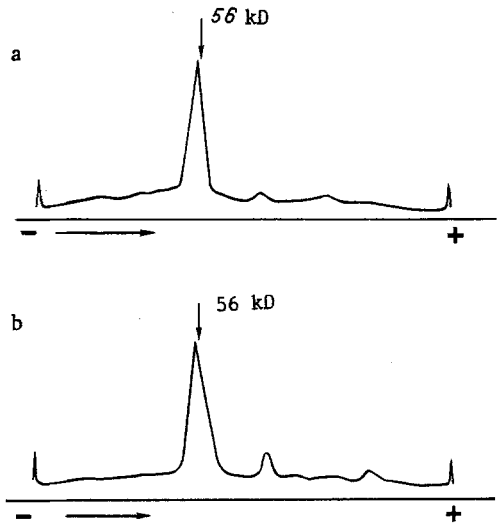

Fig. 1. SDS-PAGE densitograms of R2 II, isolated by affinity (a) and immunoaffinity (b) chromatography.

concentrating and 10% separating gels. Protein was determined by the method in [10] with a solution of Coomassie G-250, DEME, sodium bicarbonate, heat-inactivated fetal calf serum, L-glutamine, gentamicin, sodium pyruvate, 50 × solution of GAT,* a 50 × solution of GT,† and phosphate buffer were obtained from Flow Laboratories (England); globulin-free horse serum from Gibco (USA), Freund's adjuvant and Bactoagar from Difco (USA), a conjugate of rabbit immunoglobulins with peroxidase, and Pristan from Sigma (USA), Coomassie G-250 brilliant blue, PMSF, Tween-20, ABTS, and bovine serum albumin (BSA) from Serva (Sweden), polyethylene-glycol 4000 from Merck (West Germany), sodium dodecyl sulfate from Ferak (West Germany), and Synpor filters (Czechoslovakia).

EXPERIMENTAL RESULTS

Altogether 12 hybridomas producing specific MCA to RII were obtained, and from them three stable clones were selected (1E6, 2A10, and 3E9), giving high antibody titers (Table 1). Work on the MCA was done in ascites fluid from BALB/c mice and in culture medium. To obtain ascites tumors hybrid cells ($5 \cdot 10^6$-$5 \cdot 10^7$) were injected intraperitoneally into syngeneic mice, sensitized beforehand by intraperitoneal injection of 0.5 ml of 2,6,10,14-tetramethylpentadecane (Pristan). MCA were isolated by precipitation in ammonium sulfate solution up to 40% saturation and kept under a layer of this same solution at 4°C (Fig. 1a).

By our modified method of isolation of RII by means of stepwise elution with DEAE-Sephadex A50 and buffer with varied ionic strength it is possible to obtain a homogeneous preparation of RII with mol. wt. of 56 kilodaltons (kD) (Fig. 1a). However, this method is time consuming and quite laborious. It must also be pointed out that during isolation the RII is subjected to considerable proteolysis. In brain preparations endogenous proteolytic degradation leads to the formation of at least three fragments with mol. wt. of 41, 39, and 37 kD respectively. To accelerate the process of enzyme isolation, we used the method of immunosorption of RII on all MCA base. MCA are known to have unique properties of specificity and affinity, and preparative quantities of antibodies can be obtained in a comparatively short time. It was expected that the use of MCA as immunosorbent would result in a highly effective method of isolation of RII in preparative amounts.

To prepare the immunosorbent we took MCA of clone 2A10, which possessed the greatest productivity: a titer of up to 1:6.5 million (Table 1). After dialysis against 10 mM Tris-HCl buffer, pH 8.6, the MCA were purified on a column with protein A-sepharose, equilibrated with the same buffer with 50 mM NaCl. Elution was carried out with 0.2M glycine buffer, followed by dialysis against 0.1M phosphate buffer, pH 7.4.

The immunosorbent was prepared on the basis of a commercial preparation of cyanogen bromide-activated sepharose 4B. The purified MCA were mixed in a suspension of the gel (5-10 mg/ml gel) for 2 h on a shaker at room temperature. Nonspecific sorption was blocked by 0.2 M glycine buffer, pH 8 for 2 h at room temperature, or overnight at 4°C. The quantity of bound MCA was determined spectrophotometrically (3 mg/ml).

*L.-Glutamic acid–L-alanine–L-tyrosine.
†L–Glutamic acid–L-tyrosine.

TABLE 2. Isolation of Regulatory Subunit II of PK from Pig Brain

State of isolation	Protein, mg	Yield, mg/kg tissue	Purification (as protein)
Supernatant (105,000 g)	5040	—	1
Immunoaffinity sorbent	1.08	2.7	4574

The pig brain was homogenized in the ratio 1:3 in buffer containing 10 mM Tris, 2 mM EDTA, 4 mM mercaptoethanol, and 1000 mM NaCl, pH 6.8-7.4 (buffer A). The homogenate was centrifuged for 15 min at 105,000 g. The supernatant was acidified with acetic acid to pH 5.2-5.5, PMSF was added to inhibit proteases, and the sample was centrifuged under the same conditions. After adjustment of the pH of the supernatant to neutral with Tris, it was applied to a column with the immunosorbent (1 × 7 cm), previously equilibrated with buffer A, at the mean rate of 30 ml/h at room temperature. If the volume of the solution exceeded 0.5 liter, salting out with 70% ammonium sulfate was used, followed by application to the immunosorbent after dialysis and centrifugation. Protease inhibitors were added to the solution. The column was washed at 4°C with 5 volumes of 100 mM K-phosphate buffer, pH 7.4, and with 2 volumes of 200 mM carbonate buffer, pH 8.2, with 100 mM acetate buffer, pH 4, against the carbonate buffer, and again with K-phosphate buffer, containing 500 mM NaCl and 0.5% Tween, pH 7.4. Protein was eluted with 8 M urea, pH 7.0. The RII was completely eluted in 1 volume of the column. The eluate was dialyzed against 0.1 M ammonium bicarbonate overnight. The reason why 8 M urea was used to elute RII from the immunosorbent was that RII is resistant to urea and it fully restored cAMP-binding activity after removal of the urea by dialysis [6].

The resulting RII preparation was tested by polyacrylamide gel (PAG) electrophoresis with sodium dodecyl sulfate and its cAMP-binding activity was determined. The RII was purified by 4574 times on the immunosorbent (Table 2; Fig. 1b). The resulting RII preparation was virtually homogeneous (Fig. 1b) and possessed high cAMP-binding activity (19.4 nmoles/mg). The MCA-based immunosorbent was used for repeated isolation of RII more than 5 times without any appreciable loss of binding capacity, and it was kept in buffer with sodium merthiolate (0.005%).

The method of osilation of RII on an immunoaffinity sorbent, developed by the writers, can thus yield a homogeneous preparation of intact RII uncontaminated by proteolytic fragments, and its purification time is reduced to 1-2 days.

LITERATURE CITED

1. J. D. Corbin, S. L. Keely, and C. R. Park, J. Biol. Chem., 250, 218 (1975).
2. J. D. Corbin, P. H. Sugden, L. West, et al., J. Biol. Chem., 253, 3997 (1978).
3. A. G. Gilman, Proc. Natl. Acad. Sci. USA, 67, 305 (1970).
4. G. Köhler and C. Milstein, Eur. J. Immunol., 6, 511 (1976).
5. E. G. Krebs, Curr. Top. Cell Reg., 5, 99 (1972).
6. O. Ouchterlony, Immun. Chemie, Berlin (1965), pp. 13-35.
7. E. M. Reiman, D. A. Walsh, and E. G. Krebs, J. Biol. Chem., 246, 1986 (1971).
8. C. S. Rubin, J. Erlichman, and O. M. Rosen, Methods Enzymol., 38, 308 (1974).
9. E. S. Severin, M. W. Nesterova, N. N. Gulyaev, et al., Adv. Enzyme Reg., 14, 408 (1976).
10. T. Spector, Anal. Biochem., 86, 142 (1978).
11. D. A. Walsh and E. G. Krebs, Enzymes, New York (1973), Vol. 8, pp. 555-581.