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Presence of matrix-specific antibodies in affinity-purified polyclonal antibodies

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In general, antigen affinity columns made with commercially prepared activated affinity supports bind antibody specific for the coupled antigen. Nonetheless, in some cases affinity purification may yield antibodies to molecules other than the molecule of interest. In this report, we demonstrate such an occurrence: an antibody which adsorbs to an Affi-Prep 10 affinity matrix was found in the serum of sheep immunized against calmodulin. The contaminating antibody bound to cell nuclei and condensed chromosomes; the composition of the Affi-Prep 10 matrix suggests that the antibody may cross-react to the sugar-phosphate backbone of DNA. We were able to remove the contaminating antibody from the anti-calmodulin by passing the affinity-purified mixture over an antigen-free Affi-Prep 10 column.

Key words: Chromatography, antigen affinity; Affi-Gel; Affi-Prep; Anti-nuclear antibody; Anti-DNA antibody; Calmodulin; (Antibody)

Introduction

Column affinity chromatography has been used for many years as a method to separate macromolecules. A typical affinity column consists of a ligand which will bind the molecule of interest coupled to a matrix which, in theory, has no affinity for the remaining molecules in the mixture. Ideally, coupling of the ligand to the affinity matrix should not interfere with its ability to bind the molecule of interest.

Although early attempts to use affinity chromatography for the purification of proteins met with only moderate success (Lerman, 1953; Arsenis and McCormick, 1966), the technique became more widely used when methods became available for coupling proteins to agarose and polyacrylamide derivatives at variable distances from the affinity matrix (Axen et al., 1967; Porath et al., 1967; Cuatrecasas et al., 1968; Cuatrecasas, 1970).

Because of the strength and specificity of the antigen-antibody interaction, affinity chromatography is particularly well suited to antibody purification. The description by Kristiansen of affinity purification of antibodies to blood proteins was one of the first examples of this method (Kristiansen et al., 1969). Antigen affinity columns can now be easily made by coupling the antigen to one of several commercially available activated matrices such as Affi-Gel 10 and 15 (Bio-Rad Laboratories, Richmond, CA), Affi-Prep 10 (Bio-Rad) or cya-

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Abbreviations: BSA, bovine serum albumin; CaM, calmodulin; EGTA, ethylene glycol bis-(β-amino ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline; Tris, tris(hydroxymethyl)aminomethane.

nogen bromide activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ).

In general, antigen affinity chromatography provides an efficient method for purification of antibodies from immune serum. However, although the affinity matrix is designed to have minimal non-specific protein binding, the components of the affinity matrix have the theoretical potential to bind immunoglobulins specifically. If such an antibody were present in immune serum, it would co-elute with the antibody of interest.

In this report we describe an instance in which the immune serum from sheep immunized with calmodulin (CaM) contained antibody which adsorbed to an Affi-Prep 10 affinity matrix, and describe a simple method to remove the contaminating antibody.

Materials and methods

Reagents

Calcium chloride, monobasic potassium phosphate, sodium acetate and dibasic sodium phosphate were obtained from Baker Chemical Co., Phillipsburg, NJ. Affi-Prep 10 and Affi-Gel 15 were obtained from Bio-Rad Laboratories, Richmond, CA. Glutaraldehyde was obtained from Eastman Kodak Co., Rochester, NY. Ammonium sulfate, boric acid, potassium chloride, sodium azide and sodium tetraborate were obtained from Fisher Chemical Co., Pittsburgh, PA. BSA, EGTA, ethanolamine, Freund's complete and incomplete adjuvants, glycine and Tris were obtained from Sigma Chemical Co., St. Louis, MO.

Calmodulin purification

All procedures were carried out at room temperature except where noted.

CaM was purified from bovine testis by a method based on the Ca^{2+}-dependent affinity of CaM for phenyl-Sepharose (Gopalakrishna and Anderson 1982). Calmodulin undergoes a conformational change upon binding calcium which results in the exposure of a hydrophobic domain (LaPorte et al., 1980); in the presence of Ca^{2+}, CaM has an increased affinity for hydrophobic molecules such as phenyl-Sepharose.

Briefly, de-tunicated bovine testes were homogenized in 1 : 2 w/v 50 mM Tris, 1 mM EGTA pH 7.5 (Tris-EGTA). 200 ml aliquots of the homogenate were heated in a microwave to 90 °C, then immediately cooled on ice. Denatured protein was removed by centrifugation. Ammonium sulfate was added to the supernatant fluid to a concentration 40% of saturation, then precipitated protein was removed by centrifugation. Ammonium sulfate was added to the supernatant to bring the concentration of ammonium sulfate to saturation and precipitated protein was collected by centrifugation. Precipitated protein was resuspended in a volume of 50 mM Tris, 1 mM $CaCl_2$, pH 7.5 (Tris-Ca^{2+}) that was one-tenth the volume of the original tissue homogenate, and dialysed against the same buffer. The protein solution was applied to a phenyl-Sepharose (Pharmacia) column equilibrated with Tris-Ca^{2+} buffer. The column was washed with approximately 20 column volumes of Tris-Ca^{2+}, then CaM was eluted with Tris-EGTA. Fractions containing protein were dialysed against 5 mM ammonium bicarbonate and lyophilized. Protein purity was evaluated by comparing the UV absorbance spectrum against published values (Klee, 1977), and by one dimensional gel electrophoresis.

Immunization

For the primary sheep used in this study, CaM was prepared for immunization by mixing 1 mg CaM with BSA (4 : 1 molar ratio) and cross-linking in the presence of 7 mM glutaraldehyde in a volume of 3 ml for 24 h at room temperature. Following dialysis against several changes of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.2) one half of the cross-linked CaM mixture was mixed with an equal volume of Freund's complete adjuvant and injected intradermally at several sites into a sheep. 3 weeks later, a boost mixture consisting of the remaining CaM mixture and an equal volume of Freund's incomplete adjuvant was injected intradermally at several sites. A final boost consisting of 1 mg native CaM in Freund's incomplete adjuvant was done at 6 weeks. Two other sheep, which had been immunized with performic acid-oxidized CaM as described by Van Eldik and Watterson (1981), were also used.

The sheep used in this study were housed at the Sheep Research Facility of the Reproductive Sciences Program at the University of Michigan, and cared for in compliance with PHS policy on humane care and use of laboratory animals.

Preparation of affinity columns

Affi-Prep 10 affinity columns were prepared according to the manufacturer's instructions. The Affi-Prep gel was washed with 10 vols. of 10 mM sodium acetate, pH 4.5. For the CaM affinity column, 15 mg CaM, at a concentration of 3 mg/ml in 10 mM Hepes, pH 7.0 was mixed with 3.0 ml of gel and incubated on a rotary shaker for 1 h at room temperature. The antigen-free column was made by incubating 5 ml of 10 mM Hepes, pH 7.0 with 4.0 ml of gel. For each column, unreacted sites were blocked by adding 0.1 ml of 1 M ethanolamine HCl, pH 8.0 per ml of gel. Following incubation on a rotary shaker for 1 h, the gel was placed in a column and washed with PBS until the OD_{280} baseline was stable. The column was equilibrated with 0.02% sodium azide in borate buffer (100 mM boric acid, 25 mM sodium tetraborate, 75 mM NaCl, pH 8.3).

Antibody purification

Antibody was purified from blood obtained from each of three CaM-immunized sheep. Blood was collected into donor bags containing EDTA (final concentration 1 mg/ml plasma). Following removal of formed blood elements by centrifugation, serum was prepared from the plasma by adding Ca^{2+} to yield a final concentration of 3 mM, and incubating at 37°C until clotting was complete. Saturated ammonium sulfate was added 1:1 (v/v) to the serum to yield an ammonium sulfate concentration 50% of saturation. Precipitated protein was collected by centrifugation, resuspended in 0.5 vol. of borate buffer and dialysed three times against 20 vols. of borate buffer. Aliquots of this solution, to which EGTA was added (final concentration 1 mM), were applied to the CaM affinity column. The column was washed with twenty column volumes of borate buffer containing 1 mM EGTA. Protein was then eluted with 200 mM glycine, pH 2.7; fractions with an $OD_{280} > 0.1$ were pooled and dialysed against three changes of borate buffer. The antigen affinity-purified protein was then applied to the antigen-free column. The column was washed, unbound protein collected, and bound protein eluted as above. A schematic diagram of this procedure is shown in Fig. 1.

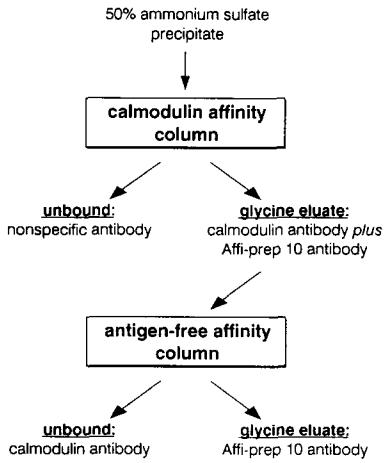

Fig. 1. Schematic diagram of calmodulin antibody purification procedure. Calmodulin affinity chromatography yields a mixture of calmodulin and chromosomal antibodies. Application of the mixture to an antigen-free Affi-Prep 10 column resolves the mixture: the calmodulin antibody remains unbound and the chromosomal antibody binds to the column and can be eluted with glycine.

Tissue culture and immunofluorescence

Rat pulmonary endothelial cells, obtained from Dr. James Varani, Department of Pathology, University of Michigan Medical School, Ann Arbor, MI were grown on 11 × 22 mm glass coverslips (VWR Scientific, San Francisco, CA) in Eagle's modified essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Whittaker Bioproducts, Walkersville, MD). The coverslips were processed for indirect immunofluorescence as described by Welsh (1983). A fluorescein labeled rabbit anti-sheep-IgG (Organon Teknika, Durham NC) was used as the secondary antibody.

Microscopy

Cells were viewed and photographed on a Leitz Aristoplan microscope with a 63 ×, 1.4 NA plan apo objective (E. Leitz, Rockleigh, NJ). Photographs were taken on Kodak Tri-X film; photographic exposure was controlled by a Leitz

Variophot/Orthomat 35 mm photomicrography system. Film was developed with Kodak HC-110 developer and negatives were printed on Kodak Polycontrast III RC paper.

Results

We have been successfully preparing polyclonal antibody to CaM from CaM-immunized sheep by antigen affinity chromatography for many years (Dedman et al., 1978; Welsh et al., 1978). However, we have often found that after an initial period of production of satisfactory antibody, subsequent bleeds from the same sheep yielded antibody which contained significant reactivity to nuclei and to condensed chromosomes in mitotic cells.

In order to investigate the phenomenon further, a new affinity column was made using an Affi-Prep 10 matrix instead of Affi-Gel 15, the matrix which had been used previously. The antibody which eluted from the Affi-Prep 10 column gave an immunofluorescence pattern which was identical to that obtained with the old Affi-Gel 15 column: mitotic cells contained significant chromosomal localization and comparatively little of the mitotic spindle localization expected for a calmodulin antibody (Fig. 2*b*). In addition, nuclei of interphase cells were often labeled (not shown). Chromosomal immunofluorescence was not seen with preimmune serum (Fig. 2*a*) or with secondary antibody alone (not shown).

In order to test the hypothesis that the immune serum contained an antibody which bound specifically to the affinity matrix a second, antigen-free, affinity column was prepared using the Affi-Prep 10 matrix. This column was made identically to the calmodulin affinity column with the exceptions that no calmodulin was added to the coupling mixture, and the column volume was roughly 25% larger. The antibody that had been eluted from the calmodulin affinity column was applied to the antigen-free column. Roughly 5% of the protein applied to the column did not bind, and was collected in the fractions which followed the void volume. The immunofluorescence patterns seen with the unbound antibody in mitotic cells were essentially identical to the calmodulin distributions previously seen using immunofluorescence techniques (Welsh et al., 1978) and in microinjection experiments using a fluorescent CaM analog (Zavortink et al., 1983). A typical metaphase mitotic cell is shown in Fig. 2*c*.

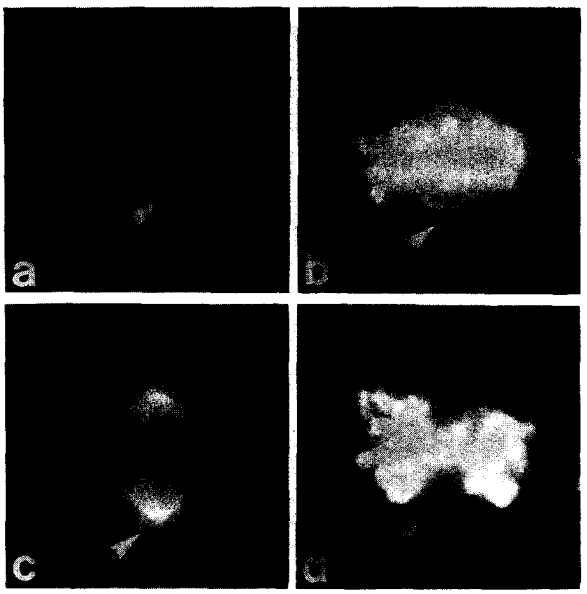

Fig. 2. Immunofluorescence patterns in metaphase mitotic rat pulmonary endothelial cells. In each panel, the arrow indicates the region of one half-spindle which would be expected to bind calmodulin antibody. *a*: preimmune serum from a sheep which was subsequently CaM-immunized. *b*: antibodies eluted from the Affi-Prep 10-calmodulin antigen affinity column. *c*: CaM antibody, which did not bind to the antigen-free Affi-Prep 10 column. *d*: chromosomal antibody, eluted from the antigen-free Affi-Prep 10 column.

The Affi-Prep 10 antigen-free column also removed contaminating chromosomal antibody from CaM antibody prepared from the serum of two other sheep (immunized against performic acid-oxidized CaM) by antigen affinity purification using an Affi-Gel 15-calmodulin affinity column (not shown).

Finally, when the protein which remained bound to the antigen-free affinity column was eluted, the antibody obtained gave a chromosomal immunofluorescence pattern similar to that eluted from the calmodulin affinity column (Figure 2*d*). However, in contrast to the antibody eluted from the calmodulin affinity column, the antibody eluted from the antigen-free column did not show any localization typical of a calmodulin antibody.

Discussion

We have found that the immune sera of sheep immunized against CaM can contain antibody which binds to Affi-Gel 15 or Affi-Prep 10, activated matrices commonly used to prepare antigen affinity columns. This contaminating antibody, which shows specific immunofluorescence localization to nuclei and condensed chromosomes, then co-elutes with the CaM antibody. The contaminating antibody can be removed by passing the mixture of CaM and chromosomal antibodies over an antigen-free column made with Affi-Prep 10. The term 'chromosomal antibody' has been used to denote the polyclonal immunoglobulin protein which binds to the antigen-free column, with the recognition that this mixture could be comprised of antibodies recognizing more than one cellular protein.

Our observations suggest that Affi-Gel and Affi-Prep may be structurally similar to some component of nuclei and condensed chromosomes. Although Affi-Gel Blue has been used in purification schemes involving nucleotide binding proteins (Pompon and Lederer, 1978; Kapp and Hubbard, 1984), it is likely that these methods depend on binding to the Cibacron blue F3GA moiety coupled to the agarose matrix (Kumar and Krakow, 1977) rather than the matrix itself. More recently, Hutchins and Porath (1986) reported adsorption of immunoglobulins to a thioether-agarose matrix. They described binding of immunoglobulins, independent of idiotype, with a 'class spectrum' broader than that of Protein A. It is possible that the polyether polymer matrix of Affi-Prep 10 could show similar, nonspecific binding of immunoglobulins. However a nonspecific interaction cannot account for the differential affinity of the CaM and chromosomal antibodies for the antigen-free column.

We are not aware of any report which describes an antibody with specific affinity to a ligand-free Affi-Prep 10 or Affi-Gel 15 matrix. Because Affi-Gel is based on a cross-linked agarose matrix and Affi-Prep is based on a hydroxylated polyether polymer matrix, one could speculate that the antibody which binds to the affinity matrices cross-reacts with the sugar-phosphate backbone of DNA.

Our results also raise the question of why antibody cross-reacting to Affi-Gel 15 and Affi-Prep 10 appears in serum from sheep immunized with CaM. The observations that preimmune serum from the same sheep does not contain the chromosomal antibody, and that the same phenomenon has occurred with several sheep suggest that the process of immunizing sheep against CaM may be responsible in some way for eliciting the production of the contaminating antibody. It is not likely that the adjuvant alone is responsible, because we have not seen chromosomal antibody contamination of antigen affinity-purified antibody obtained from sheep immunized against other proteins using the same adjuvant and immunization schedule. The glutaraldehyde induced cross-linking to BSA is not likely to have contributed to the phenomenon because chromosomal antibody was found in the sera of sheep immunized against performic acid-oxidized CaM. Therefore, it appears that CaM, or some trace contaminant of the CaM used for immunization, is responsible for eliciting the production of the chromosomal antibody.

In spite of our incomplete understanding of the genesis and specificity of the contaminating antibody, the fact remains that the potential for co-purification of such antibody exists. The use of an antigen-free column is a simple method to remove such contaminants; this technique should be considered when a polyclonal antibody purified by conventional affinity chromatography appears to recognize proteins other than the original immunogen.

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Affi-Gel and Affi-Prep are registered trademarks of the Bio-Rad Corporation. Sepharose is a registered trademark of the Pharmacia Corporation.

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