

Localization of the Catalytic Subunit of Cyclic AMP-dependent Protein Kinase in Cultured Cells Using a Specific Antibody

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ABSTRACT We developed a specific antibody to the catalytic subunit (C-subunit) of cyclic AMP-dependent protein kinase and used it to localize C-subunit in cultured cells. C-subunit antigen was purified from bovine cardiac muscle and cross-linked to hemocyanin with glutaraldehyde. Immunized goat serum showed a low titer of antibody after boosting; it was enriched 100-fold by affinity chromatography on catalytic subunit-Sepharose. The antibody immunoprecipitated C-subunit from type I and type II holoenzyme and depleted enzymatic activity from solution. At 12.5 nM antigen, 1 μ g antibody immunoprecipitated 10 ng of C-subunit. Immunoprecipitation of ^{35}S -labeled cell extracts and ^{125}I -antibody detection on nitrocellulose paper revealed that the antibody specifically reacts with C-subunit in Chinese hamster ovary (CHO) whole cell extracts. Using indirect immunofluorescence to localize C-subunit, we found a pattern of diffuse staining in the cytoplasm of CHO cells with little or no nuclear staining. A similar distribution of the enzyme was observed in Swiss 3T3 cells, bovine endothelial tracheal cells, human lung fibroblasts and NRK cells. Treatment of CHO cells with 8-bromo-cyclic AMP produced no change in the pattern or intensity of immunofluorescence. We conclude that the majority of C-subunit is localized in cytoplasm and that in cultured fibroblasts exposure to cyclic AMP analogues causes no apparent redistribution of catalytic subunit.

Cyclic AMP (cAMP) is thought to act in eucaryotic cells through protein phosphorylations catalyzed by the catalytic subunit of cAMP-dependent protein kinase. Cyclic AMP-dependent protein kinase holoenzyme consists of two regulatory and two catalytic subunits. The association of these subunits represses the activity of the catalytic subunit. When cAMP binds to the regulatory subunits, the catalytic subunits are released and become active.

In cultured or isolated cells cAMP-dependent protein kinase has been implicated in a variety of processes which are affected by cAMP. Exogenous cAMP reverses the phenotypic expression of transformation in fibroblastic cells (1-3). Mutant CHO cells containing defects in cAMP-dependent protein kinase, however, no longer respond to cAMP and continue to maintain the transformed morphology (4-6). Specific cAMP-dependent protein kinase phosphorylation of cytoskeletal proteins involved in motility has been described in several laboratories (7-11). In the process of phagocytosis, type I regulatory subunit has been associated with newly phagocytosed granules in neu-

trophils (12).

Because cAMP-dependent protein kinase mediates the phosphorylation of a wide variety of substrate proteins, it is not clear how extracellular signals acting through cAMP are able to elicit specific responses within the cell. One mechanism that would allow cAMP-dependent protein kinase to act selectively involves compartmentation of the holoenzyme or the free catalytic subunit into discrete intracellular pools. Such a subcellular localization and redistribution of kinase subunits has been demonstrated in several biochemical studies (13-17).

An immunocytochemical approach that avoids some of the artifacts associated with biochemical studies (18-20) has also been used to examine this issue (21). Browne et al. (22) localized the regulatory subunits of cAMP-dependent protein kinase in cultured cells using antisera against bovine type I and type II regulatory subunits. These studies demonstrated selective association of type II regulatory subunit with the mitotic spindle of PtK₁ cells.

A more complete understanding of the protein kinase system

in cultured cells should be obtained by identifying the distribution of both regulatory and catalytic subunits. Therefore, we prepared an antibody directed against the catalytic subunit of cAMP-dependent protein kinase. Because the catalytic subunit is present in cells in low concentrations and is a poor antigen we have extensively characterized the anticatalytic subunit antibody. It was found to recognize free catalytic subunit and cAMP-dependent protein kinase holoenzyme. Immunofluorescence studies showed the enzyme was predominantly concentrated in the cytoplasmic fraction of fibroblasts but did not redistribute after exposure to cAMP analogues.

MATERIALS AND METHODS

Antibody Production

Catalytic subunit of cAMP-dependent protein kinase (predominantly type II) was purified from bovine heart tissue by the method of Peters et al. (23). After gel filtration the final product appeared as a single band on a 10% SDS acrylamide slab gel and its phosphotransferase activity was 3.4 $\mu\text{mol}/\text{min}/\text{mg}$ protein using the histone kinase assay of Corbin and Riemann (24).

Antigen was prepared by preparative electrophoresis of catalytic subunit in a 5% SDS acrylamide slab gel. Catalytic subunit was eluted from the gel by electrophoresis, dialyzed against water and combined with a three- to six-fold molar excess of keyhole limpet hemocyanin. After lyophilization, the resultant powder was suspended in 400 μl of acetone containing 0.2% glutaraldehyde and allowed to react for 3 h at room temperature. The reaction was stopped with a 10-fold molar excess of ethanolamine (pH 8.0). The acetone was evaporated and the insoluble aggregate was suspended in Dulbecco's PBS and emulsified in complete Freund's adjuvant. A goat was injected with 0.5-mg hemocyanin-conjugated catalytic subunit and boosted at 3–4-wk intervals with 0.25 mg of antigen. Sera were collected 14 d after each boost. The immunoglobulin fraction was purified from whole serum by precipitating three times with 50% saturated ammonium sulfate.

Anticatalytic subunit antibodies were purified from goat immunoglobulin by affinity chromatography. A column containing 7 mg native catalytic subunit on 7 ml of CNBr-activated Sepharose 4B was prepared according to March et al. (25) and was stored in 0.1 M borate–0.5 M NaCl buffer (pH 8) containing 0.1% bovine serum albumin (BSA) and 0.02% sodium azide (26). To purify antibody, up to 800 mg immunoglobulin was passed over the column; the column was washed with borate buffer (pH 8) followed by 0.1 M sodium acetate–0.5 M NaCl (pH 4.8) until the OD of the effluent was less than 0.1. Anticatalytic subunit antibody was eluted with 0.1 M acetic acid–0.5 M NaCl (pH 2.8) and stored in 20 mM sodium phosphate–150 mM NaCl (pH 7.3).

Immunoprecipitations

Immunoprecipitation of holoenzyme was carried out in 10 mM sodium phosphate (pH 7.3), 100 mM NaCl containing 10 μg of antigen and 30 μg of immunoglobulin in a final volume of 550 μl for 30 min at 4°C. 25 μl of *Staphylococcus aureus* (resuspended in PBS, pH 7.3, after boiling in SDS [27]) was added and agitated occasionally for 30 min. The *S. aureus* was then pelleted at 14,000 g for 2 min and resuspended in 40 μl of buffer containing 2% SDS, 10% mercaptoethanol, 10% glycerol, 20 mM sodium phosphate (pH 6.8), and 0.001% bromophenol blue. Samples were boiled 3 min, the *S. aureus* repelleted and the resulting supernatant subjected to electrophoresis on a 7.5% discontinuous acrylamide gel (28). After electrophoresis, the gel was fixed and stained with 0.25% Coomassie Blue in 50% trichloroacetic acid and destained in 7% acetic acid.

Antibody-induced inhibition or depletion of C-subunit enzymatic activity from solution was carried out essentially as described by Schwoch et al. (29). Preimmune or affinity-purified immunoglobulin was incubated overnight in 40 μl of Dulbecco's PBS containing 5 mM mercaptoethanol, 40 μg of BSA and up to 2.5 pmol of holoenzyme or catalytic subunit. Loss of activity by depletion was measured after precipitation of immune complexes with 20 μl of *S. aureus* prepared as described above, using the filter paper (24) or phosphocellulose paper (30) assay.

To immunoprecipitate catalytic subunit from Chinese hamster ovary (CHO) cells, wild-type or 10260 kinase-deficient mutant cells were grown in 35-mm tissue culture dishes and metabolically labeled for 4 h with 200–600 $\mu\text{Ci}/\text{ml}$ of ^{35}S -methionine in methionine-free Dulbecco-Vogt media. The cells were then washed three times with 2 ml of 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA and scraped off the dishes into 0.5 ml of solution containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS (RIPA buffer; 31). Immunoprecipitations were carried

out in RIPA or PBS buffer containing 1% BSA, cell extract containing 3×10^7 cpm of radioactivity, and 30–50 μg of antibody in a total volume of 0.5–1 ml for 30 min at 4°C. 100 μl of *S. aureus* in RIPA buffer containing 1% BSA was added for 30 min at 4°C. *S. aureus* pellets were obtained and washed three times with 1 ml of RIPA buffer, then solubilized and electrophoresed as described above. After fixation and staining the gels were dehydrated with dimethylsulfoxide, infiltrated with 2,5-diphenyloxazole, dried, and fluorographed (32).

^{125}I -Antibody Immunodetection

In the first step of this method 500 μg of cell extract was incubated with 50 μg of antibody for 2 h in 600 μl of 50 mM sodium phosphate, pH 7.6, containing 0.1% SDS. 100 μl of *S. aureus* in Tris-buffered saline, pH 7.5, was added and agitated. After 30 min it was pelleted; the pellet was washed with 1 ml of Tris-buffered saline, solubilized in SDS gel buffer, and repelleted. The supernatant was electrophoresed on a 7.5% discontinuous gel containing 7.5% glycerol and 0.2% linear polyacrylamide.

After electrophoresis the gel was rinsed for 10 min in 25 mM Tris, 192 mM glycine, pH 8.3, 20% methanol. Proteins were then transferred from the gel by horizontal electrophoresis onto nitrocellulose paper (Bio-Rad Laboratories, Richmond, CA) for 15 h using a Canalco CD-2 power source (Miles Laboratories, Inc., Research Product Div., Elkhart, IN). The transfer was carried out in a chamber from Custom Crafting (Houston, TX). The desired lanes were cut from the paper and treated with 3% BSA, 0.1% goat IgG, 0.1% human IgG in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA for 1 h. 5×10^5 cpm/lane of ^{125}I -affinity purified anti-C antibody labeled by the chloramine T method was then added and incubation continued for 1 h. The papers were then washed exhaustively in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA containing 0.5% Triton X-100, 0.25% gelatin, and 0.1% SDS. Papers were then rinsed in water, dried, and exposed to Kodak X-OMAT XR-5 film.

To detect catalytic subunit in rat heart tissue, samples solubilized in SDS gel buffer were fractionated on a 10% acrylamide gel containing 0.13% bis-acrylamide (33) and blotted onto nitrocellulose paper as described above for 3 h. Unreacted binding sites on the paper were blocked by incubation in 5% BSA in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone. 10^6 cpm/ml of iodinated antibody was added and incubation was continued for 3 h. The papers were washed as described above, dried, and autoradiographed on a Kodak X-OMAT XAR film.

Preparation of Holoenzymes

Purified regulatory subunits from type I and type II holoenzymes were treated with urea to remove cAMP (34) then recombined with the respective catalytic subunit from type I or type II holoenzyme (34, 35). Reconstituted holoenzyme was separated from unreacted catalytic subunit by CM-Sephadex C50 chromatography in a buffer containing 20 mM 2-(*N*-morpholino)ethane sulfonic acid (MES), pH 6.8, 100 mM NaCl, 0.1 mM EDTA, and 15 mM mercaptoethanol. BSA (1 mg/ml) was added for storage.

Immunofluorescence

To localize catalytic subunit in cultured cells using indirect immunofluorescence, cells grown on glass cover slips were washed three times in PBS and fixed in 4% formaldehyde for 10 min at 37°C. Cells were rinsed in PBS and permeabilized by treatment for 6 min with methanol at -20°C and for 30 s with acetone at -20°C . After rinsing in PBS, the cells were incubated for 5 min in BSA (5 mg/ml) \pm 1 mg/ml rabbit immunoglobulin. The cells were then incubated with 10–50 μg of control serum or affinity-purified anti-C antibody for 30 min at 23°C, washed in three changes of PBS then incubated with rhodamine-labeled rabbit anti-goat IgG diluted 1:10 for 30 min at 30°C. The cells were washed in three changes of PBS, blotted dry and mounted on acid-washed microscope slides in polyvinyl alcohol. Slides were examined on a Leitz Diavert fluorescence microscope and photographed using Polaroid 084 film (ASA 3000).

Materials

All reagents for electrophoresis were from Bio-Rad Laboratories. Keyhole-limpet hemocyanin and *S. aureus* (Pansorbin) were from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA. The goat and services for antibody production were supplied by Henry Carwile, D.V.M., at Bethyl Laboratories (Montgomery, TX). CNBr-activated Sepharose 4B, CM-Sephadex C50, and Ficoll 400 were from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ. Phosphocellulose paper was supplied by Whatman Inc. (Paper Div., Clifton, NJ). Polyvinylpyrrolidone 360 was from Sigma Chemical Co. (St. Louis, MO). ^{35}S -methionine (1,000 Ci/mmol) was from New England Nuclear (Boston, MA) and Na^{125}I (15 mCi/ μg I) was from Amersham Corp. (Arlington Heights, IL). Rhodamine-labeled rabbit anti-goat immunoglobulin was from Cappel N.L. Laboratories, Inc., Cochranville, PA). Polyvinyl alcohol (Gelvatol)

was a gift from Monsanto Co. (St. Louis, MO). Purified regulatory and catalytic subunits from type I and type II cAMP-dependent protein kinase were graciously provided by Steven Smith (University of Washington). The mutant 10260 cell line from CHO cells that is deficient in cAMP-dependent protein kinase was provided by Michael Gottesman (5, 6) (National Cancer Institute).

RESULTS

Preparation of Antigen

Catalytic subunit of cAMP-dependent protein kinase was purified from beef cardiac muscle following the procedure of Peters et al. (23). Native catalytic subunit was purified by ion-exchange chromatography and gel filtration. The final purification of enzyme activity was 40,000-fold over the initial tissue extract supernatant. The purified enzyme had a specific activity of 3.4 $\mu\text{mol}/\text{min}/\text{mg}$ protein using mixed histones as substrate. SDS gel electrophoresis of the purified enzyme revealed a single band with subunit M_r of 40,000 (Fig. 1A).

To prepare antigen for immunization, the catalytic subunit preparation was dissolved in 1% SDS–5% mercaptoethanol and subjected to preparative electrophoresis in a 5% acrylamide gel. The catalytic subunit band was eluted from the gel and conjugated to keyhole limpet hemocyanin with glutaraldehyde. The insoluble aggregate was macerated in complete Freund's adjuvant and injected subcutaneously into a goat. The animal was boosted and serum collected as described in the Materials and Methods.

Purification of Antisera

Sera collected following the first three boosts were tested for anticatalytic subunit antibody by indirect immunoprecipita-

tion. As shown in Fig. 1B, lane 1, 1 mg of preimmune immunoglobulin produced no immunoprecipitation of catalytic subunit. Other bands in this lane on the Coomassie-Blue-stained gel represent the heavy and light chains of IgG, carrier BSA and proteins solubilized from *S. aureus*. An equal amount of immune IgG, however, precipitated approximately 1 μg of purified catalytic subunit (Fig. 1B, lane 2). The low titer of the antisera suggested that further characterization of immunoglobulin would be difficult. Therefore, anticatalytic subunit antibody was purified by affinity chromatography on a column of catalytic subunit bound to CNBr-activated Sepharose. The flow through from the affinity column did not react with catalytic subunit (Fig. 1B, lane 3), whereas 10 μg of affinity purified antibody immunoprecipitated $\sim 1 \mu\text{g}$ of antigen (Fig. 1B, lane 4). Reaction of catalytic subunit with additional antibody increased the amount of antigen which was precipitated (Fig. 1B, lane 5). We have noticed that goat IgG is not absorbed to *S. aureus* efficiently, but that immune complexes of goat IgG are adsorbed. Thus, the amount of IgG revealed on the gel by Coomassie Blue staining is proportional to the amount of catalytic subunit that is immunoprecipitated. Sera obtained following further boosts were also affinity purified by the same procedure and were assayed by immunoprecipitation of purified catalytic subunit. The antibody titer did not change appreciably after multiple boosts (data not shown).

Characterization of Anticatalytic Subunit Antibody

To characterize the anticatalytic subunit antibody we first tested its ability to precipitate type I and type II cAMP-dependent protein kinase. Type I and type II holoenzymes were prepared by reconstituting individually purified regulatory and catalytic subunits as described in the Materials and Methods. Unreacted catalytic subunit was separated from reconstituted holoenzyme by chromatography on CM-Sephadex, as shown in Fig. 2A and B. Type I holoenzyme activity was stimulated 8.3-fold by 5 μM cAMP; type II holoenzyme activity was stimulated 3.9-fold by 5 μM cAMP. In the presence of cAMP, both types of holoenzyme exhibited a specific activity of 0.6 $\mu\text{mol}/\text{min}/\text{mg}$ protein at a concentration of 10 $\mu\text{g}/\text{ml}$. Anticatalytic subunit antibody immunoprecipitated catalytic subunit from both holoenzymes (Fig. 2C and D). In Fig. 2C, lane 1 shows the SDS acrylamide gel profile of type I holoenzyme. The band at 49,000 is R_1 subunit. The doublet at 36,000–40,000 is catalytic subunit.¹ The dense band at 67,000 and the other bands in the lane represent the BSA preparation in which the holoenzyme was stored. Lane 2 in Fig. 2C shows the profile of the immunoprecipitate following reaction of holoenzyme with preimmune IgG. In addition to the heavy and light chain of IgG, a band for BSA and faint bands representing trace contamination of the immunoprecipitate with regulatory and catalytic subunits are seen. In Fig. 2C, lane 3 shows the proteins in the immunoprecipitate after incubation of type I holoenzyme with anti-C subunit antibody. In contrast to lane 2, in Fig. 2C, catalytic subunit doublet at 36,000–40,000 is specifically immunoprecipitated. No increase above background in the regulatory subunit band is detectable. Fig. 2D shows an equivalent experiment using type II holoenzyme. As seen in Fig. 2D, lane 3, anticatalytic subunit antibody reacts with the catalytic sub-

FIGURE 1 Catalytic subunit antigen and its immunoprecipitation by anti-C antibody. (A) Catalytic subunit purified from bovine heart (10 μg) was electrophoresed in a 10% acrylamide–0.1% SDS discontinuous gel and stained with Coomassie Blue. (B) 10 μg of catalytic subunit in 500 μl of 20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mg/ml BSA was incubated for 2 h at 4°C with 1 mg preimmune IgG (lane 1), 1 mg immune IgG (lane 2), 10 μg of immune IgG affinity column flow through (lane 3), 10 μg of affinity-purified antibody (lane 4), or 50 μg of affinity-purified antibody (lane 5). Immune complexes were pelleted with 1 ml (lanes 1–2) or 0.05 ml (lanes 3–5) of *S. aureus*. After washing the bound proteins were solubilized in SDS and electrophoresed on a 7.5% acrylamide gel. Proteins were detected by Coomassie-Blue-staining. BSA, carrier BSA. *h-IgG*, heavy chain of IgG. *C-subunit*, catalytic subunit. *l-IgG*, light chain of IgG.

¹ We consistently observe that purified native catalytic subunit appears as a single band on gels, but after storage a second slightly lower molecule weight band is generated.

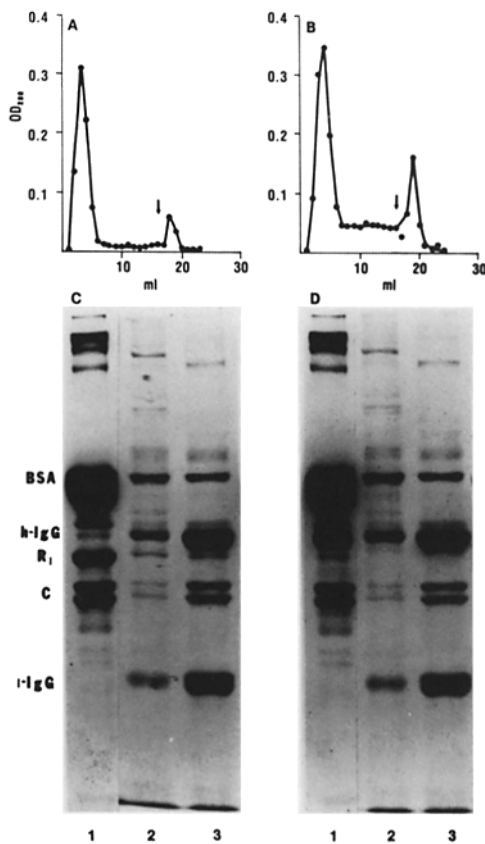


FIGURE 2 Immunoprecipitation of cAMP-dependent protein kinase holoenzymes. (A) Separation of type I cAMP-dependent protein kinase holoenzyme from catalytic subunit by chromatography on CM-Sephadex. 2.5 ml of solution containing type I holoenzyme and unreacted regulatory and catalytic subunits was passed over 2.5 ml of resin in 20 mM MES, pH 6.8, 100 mM NaCl, 0.1 mM EDTA, 15 mM 2-mercaptoethanol buffer. Type I holoenzyme eluted in the flow through. The column was then washed with buffer containing 0.5 M NaCl (arrow) and unreacted catalytic subunit was eluted, as seen in the second peak. (B) Identical profile for type II holoenzyme. (C) Immunoprecipitation of type I holoenzyme with anticycatalytic subunit antibody. Lane 1 shows reconstituted type I holoenzyme after electrophoresis on a 7.5% discontinuous SDS acrylamide gel. Coomassie Blue staining of the gel reveals type I regulatory subunit (R_1 , 49,000) and catalytic subunit (C, doublet at 40,000). The other bands represent BSA and proteins present in the BSA that was added to stabilize the holoenzyme preparations. Lane 2 shows the immunoprecipitate recovered after 10 μ g of type I holoenzyme was incubated with 30 μ g of preimmune IgG. Lane 3 shows the immunoprecipitate recovered after 10 μ g of type I holoenzyme was incubated with 30 μ g of affinity-purified anti-C antibody and sedimented with *S. aureus*. (D) Immunoprecipitation of type II holoenzyme with anticycatalytic subunit antibody. Lane 1 shows reconstituted type II holoenzyme from B in 1 mg/ml BSA after electrophoresis on a 7.5% SDS acrylamide gel. Coomassie Blue staining of the gel revealed regulatory subunit II at 54–56 kdaltons, catalytic subunit and the proteins in carrier BSA. Lane 2 shows the immunoprecipitate recovered after the incubation of 10 μ g of type II holoenzyme with 30 μ g of preimmune IgG. BSA, IgG and catalytic subunit were detected as described for lane 2 in C. Type II regulatory subunit comigrated with IgG heavy chain and could not be detected. Lane 3 show the proteins recovered from the immunoprecipitation of 10 μ g of type II holoenzyme with 30 μ g of anticycatalytic subunit antibody.

unit in type II holoenzyme. Because the regulatory subunit (Fig. 2D, lane 1) comigrated with IgG heavy chain (Fig. 2D lanes 2 and 3) it was not clear if immunoprecipitation of

holoenzyme with the antibody also caused coprecipitation of type II regulatory subunit. Since the holoenzyme preparations were devoid of free C subunit, these experiments show that the C subunit in both Type I and Type II holoenzyme was readily recognized by the antibody.

To test whether the antibody inhibited the enzymatic activity of catalytic subunit, catalytic subunit (0.5 μ g/ml, 12.5 nM) was preincubated with buffer, preimmune IgG (0.5 mg/ml) or antibody (0.5 mg/ml). A portion of the incubation solution was assayed for phosphotransferase activity (Table I). No inhibition of catalytic subunit activity by preimmune IgG was observed. The anticatalytic subunit antibody, however, produced partial but not complete inhibition of kinase activity.

We also examined the ability of the antibody to deplete enzymatic activity from solution. Solutions containing 0.5 μ g/ml catalytic subunit (12.5 nM) were preincubated with buffer, preimmune IgG (200 μ g/ml) or various concentrations of anti-C antibody (2–200 μ g/ml) at 4°C overnight. Immune complexes were sedimented by adsorption to *S. aureus* and a portion of the supernatant was assayed for phosphotransferase activity (Fig. 3). Catalytic subunit, shown by the open circle, had a specific activity of 0.13 μ mol phosphate incorporated/min/mg protein. After pretreatment with preimmune IgG, shown by the open square, the activity in the supernatant was unaffected. Increasing amounts of antibody, however, quantitatively removed catalytic activity from solution. Under these

TABLE I
Inhibition of Catalytic Subunit Phosphotransferase Activity by the Anti-C Subunit Antibody

Immunoglobulin added	Protein kinase activity μ mol/min/mg
None	1.10
Preimmune	1.18
Anti-C subunit	0.58

100 ng of catalytic subunit was incubated 15 h at 4°C in 200 μ l of PBS containing 200 μ g BSA, 5 mM mercaptoethanol and \pm 100 μ g immunoglobulin. The tubes were vortexed and a 20- μ l aliquot was assayed for histone kinase activity (30).

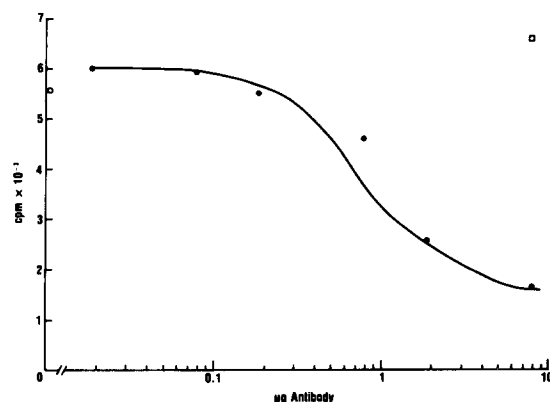


FIGURE 3 Immunodepletion of catalytic activity from solution by anticycatalytic subunit antibody. A 12.5 nM solution of catalytic subunit was incubated overnight with the antibody at concentrations of 2–200 μ g/ml. Immune complexes were removed from solution with *S. aureus* and an aliquot of supernatant was assayed for phosphotransferase activity (24). (○) Activity of catalytic subunit incubated in buffer alone. (□) Activity of catalytic subunit incubated with 200 μ g/ml of preimmune IgG. (●) Activity of catalytic subunit incubated with varying amounts of anti-C antibody.

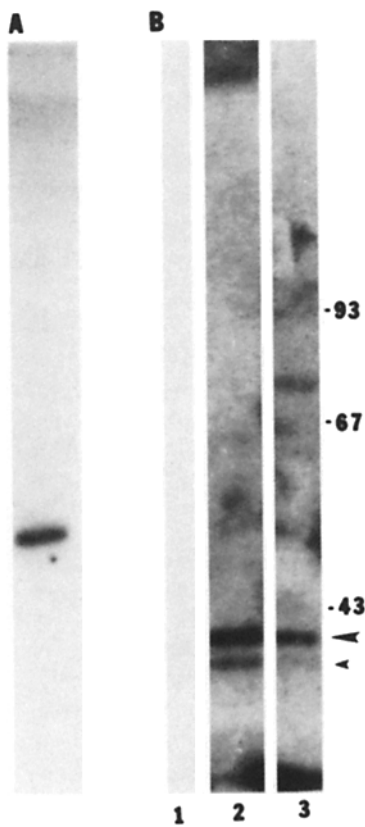


FIGURE 4 ^{125}I -Antibody immunodetection of catalytic subunit. (A) 100 μg of rat heart homogenate was fractionated on a 10% SDS acrylamide gel and transferred to nitrocellulose paper as described in Methods and Materials. The paper was incubated in 5% BSA for 90 min. 10^6 cpm/ml of ^{125}I -labeled anti-C antibody was added and the incubation was continued for 3 h. The lane shown was autoradiographed for 3 d. (B) CHO cells grown in spinner culture were solubilized in hot 1% SDS. The extracts were diluted to 0.1% SDS with 25 mM sodium phosphate buffer, pH 7.6, and incubated with 50 μg of anti-C antibody and precipitated with *S. aureus*. The pellets were electrophoresed in a 7.5% discontinuous acrylamide gel and blotted onto nitrocellulose paper. Lane 1 shows a lane stained with 5×10^5 cpm of ^{125}I -labeled

preimmune IgG. Lane 2 shows a lane stained with 5×10^6 cpm of ^{125}I -labeled anti-C antibody. In lane 3, 10 ng of purified catalytic subunit was electrophoresed, blotted and stained alongside lane 2. The numbers indicate the molecular weight markers phosphorylase a (93,000), BSA (67,000), and ovalbumin (43,000).

conditions (12.5 nM antigen), 1 μg of antibody was required to precipitate 10 ng of catalytic subunit.

To test that catalytic subunit was the only protein in cells recognized by our anti-C antibody, an iodinated anti-C antibody was used to stain homogenates of tissues or cells that were fractionated by SDS acrylamide gel electrophoresis. In the experiment depicted in Fig. 4A, 100 μg of rat heart homogenate was solubilized in SDS gel buffer and electrophoresed on a 1% SDS acrylamide gel. Proteins were transferred to nitrocellulose paper and exposed to iodinated anti-C antibody and washed exhaustively. Autoradiography revealed that the antibody reacted with a single protein migrating at 40,000 daltons. In Fig. 4B, CHO cells were solubilized in SDS and immunoprecipitated with anti-C antibody. The immunoprecipitates were then fractionated on a 7.5% SDS acrylamide gel, blotted onto nitrocellulose paper and exposed to ^{125}I -labeled pre-immune IgG (Fig. 4B, lane 1) or anti-C antibody (Fig. 4B, lane 2). Purified catalytic subunit, shown in Fig. 4B, lane 3, was electrophoresed in a parallel lane, blotted onto nitrocellulose paper and exposed to iodinated anti-C antibody alongside Fig. 4B, lane 2. This experiment shows that the antibody specifically detects catalytic subunit at M_r 40,000 and its degradation product at M_r 36,000.

The specificity of the antibody was also tested by immunoprecipitating catalytic subunit from ^{35}S -labeled CHO cell extracts. Detergent-solubilized extracts of CHO cells were incubated with either preimmune or immune antibody. Immune complexes were collected using *S. aureus* and fractionated by

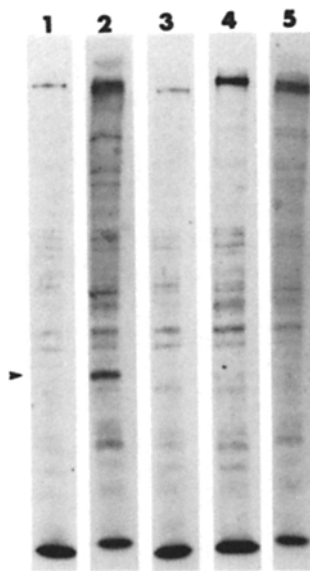


FIGURE 5 Immunoprecipitation of ^{35}S -labeled CHO cell proteins with anticatalytic subunit antibody. Wild-type (lanes 1-3) or 10260 kinase-deficient mutant (lanes 4-5) CHO cells were metabolically labeled for 4 h with 670 $\mu\text{Ci}/\text{ml}$ of ^{35}S -methionine and solubilized with RIPA buffer. 3.5×10^7 cpm of wild-type cell extract was incubated with 50 μg of preimmune IgG (lane 1), anti-catalytic subunit antibody (lane 2) or antibody preadsorbed with a fourfold molar excess of purified catalytic subunit (lane 3). 3.5×10^7 cpm of 10260 mutant extract was incubated with 50 μg of preimmune IgG (lane 4) or anti-C antibody (lane 5). Immune

complexes were precipitated with *S. aureus* and electrophoresed on a 7.5% acrylamide gel and fluorographed.

polyacrylamide gel electrophoresis. Fig. 5, lane 1 shows the fluorograph of proteins precipitated by the preimmune IgG. Numerous bands of protein nonspecifically precipitated are evident. The same pattern is obtained with experiments carried out with *S. aureus* alone and no added immunoglobulin. In Fig. 5, lane 2 shows the immunoprecipitate obtained using the anticatalytic subunit antibody. In addition to many nonspecific bands, a prominent band at 40,000 daltons (arrowhead) is also precipitated. This band coelectrophoresed with authentic cardiac catalytic subunit and is presumably ^{35}S -labeled CHO catalytic subunit. Preadsorption of the antibody with a fourfold molar excess of catalytic subunit led to the selective elimination of the 40,000 dalton band (Fig. 5, lane 3).

To insure that the 40,000 dalton band precipitated by the antibody was CHO cell cAMP-dependent protein kinase catalytic subunit, we used a mutant CHO cell line (10260) isolated by Gottesman et al. (5) that lacks cAMP-dependent protein kinase activity. As shown in Fig. 5, lane 4, preimmune IgG produced the same nonspecific precipitation as seen in wild-type cells. The anti-C antibody precipitated no 40,000 dalton band from the 10260 cell extracts (Fig. 5, lane 5).

Immunofluorescent Localization of Catalytic Subunit in Cultured Cells

To study the distribution of catalytic subunit in cultured cells, we used an indirect immunofluorescence approach. Although several methods of fixing and staining cells were tried, the procedure detailed in Materials and Methods using formaldehyde fixation followed by methanol and acetone permeabilization gave the best results. Indirect immunofluorescence experiments were performed with affinity-purified goat anti-catalytic subunit antibodies and rhodamine-conjugated rabbit anti-goat immunoglobulin.

The immunofluorescent localization of C-subunit in CHO cells is shown in Fig. 6. Fig. 6A shows CHO cells at low magnification stained with preimmune IgG (Fig. 6A) or anti-C antibody (Fig. 6B). Specific immunofluorescence is apparent in cells treated with anticatalytic subunit antibody. At higher magnification (Fig. 6C and D), the antibody appears to localize

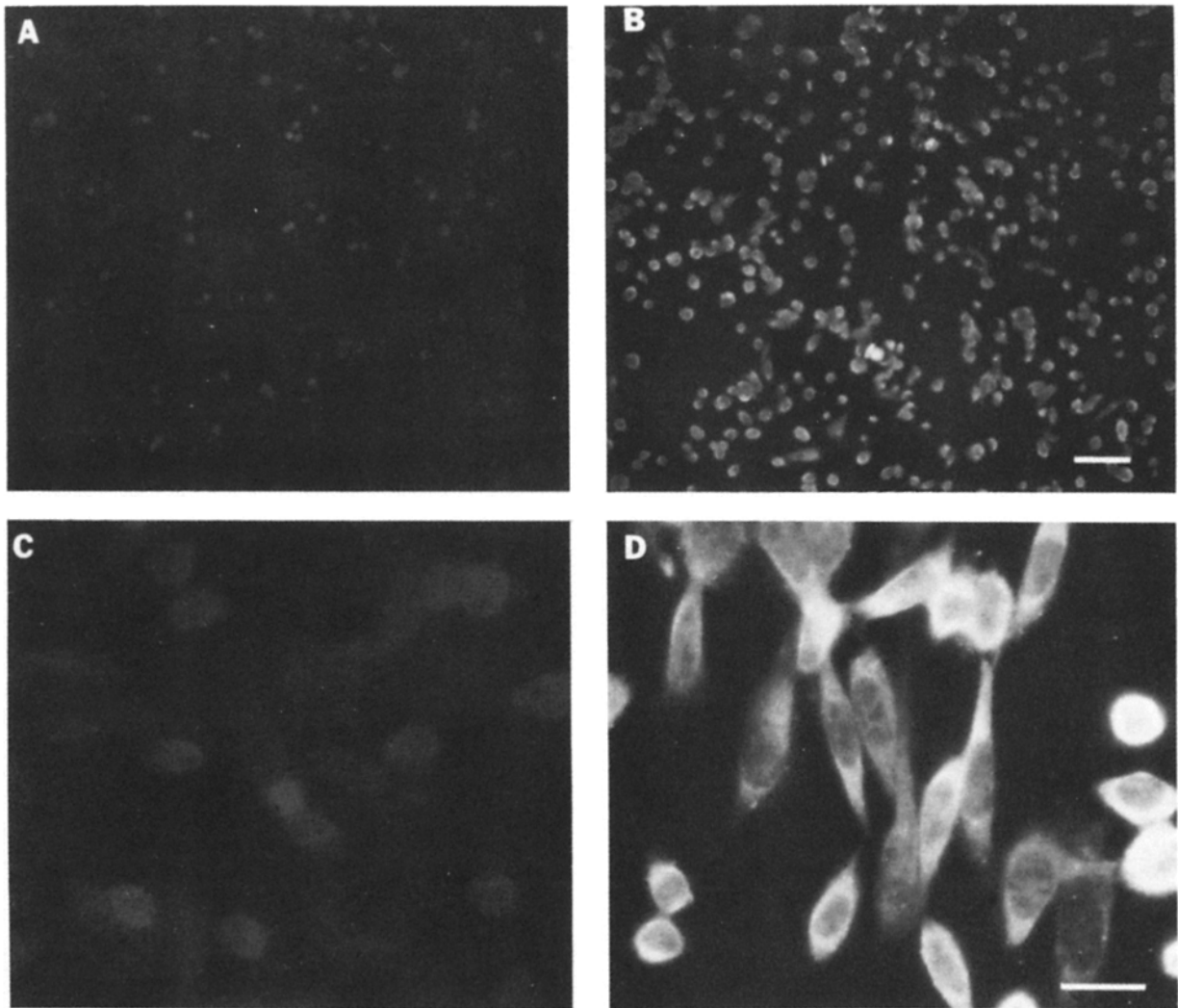


FIGURE 6 Immunofluorescent localization of catalytic subunit in CHO cells. Cells were fixed and permeabilized as described and stained with 10 μ g preimmune IgG (A and C) or anticatalytic subunit antibody (B and D) followed by rhodamine-labeled rabbit anti-goat diluted IgG. A and B, Bar, 0.1 mm. A and B staining at low magnification. Bar, 20 μ m. C and D show a portion of these fields at high magnifications.

predominantly in the cytoplasm with little or no nuclear staining. Immunofluorescent staining for catalytic subunit has also been observed in bovine endothelial trachea cells (Fig. 7A), Swiss 3T3 cells (Fig. 7B), NRK fibroblasts (Fig. 7C), and WI-38 human lung fibroblasts (Fig. 7D). These studies show for all cell types examined that catalytic subunit is distributed diffusely in the cytoplasm and is present in reduced amounts in the nucleus. We noted in both Swiss 3T3 and WI-38 cells that particularly bright fluorescence was associated with membrane ruffles. The basis for this observation is not clear but it suggests a possible association of protein kinase with areas of membrane activity.

To determine whether the distribution and intensity of immunofluorescent staining was altered by dissociation of the endogenous cAMP-dependent protein kinase holoenzyme, CHO cells were treated with 1 mM 8-bromo-cAMP for various lengths of time before fixation. As seen in Fig. 8, treatment of CHO cells with 8-bromo-cAMP for 1 to 10 min did not cause a change either in the distribution of fluorescence or in its relative intensity. Even after four hours, when the cells have produced elongated cytoplasmic processes, the staining pattern is identical to that of untreated cells (data not shown).

DISCUSSION

Characterization of the Antibody to Catalytic Subunit

We characterized a goat antibody to cAMP-dependent protein kinase catalytic subunit and used it for the immunolocalization of catalytic subunit in cultured cells. It was especially important to determine the specificity of the antibody because catalytic subunit is found at very low levels (0.01% of total cell protein) in cells and because the protein is a poor antigen.

The antigen used for immunization was purified by conventional methods. The pure protein was a single band on SDS acrylamide gels and its molecular weight and specific activity agreed with published values (26, 36, 37). As previously noted, a second lower molecular weight band appeared in the purified catalytic subunit preparation after storage. Alhanaty et al. (38) recently described a proteinase that specifically cleaves a 6,000 dalton fragment from free catalytic subunit.

The enzyme was further purified by SDS acrylamide preparative gel electrophoresis before immunization. Antisera recovered from the goat were purified by affinity chromato-

phy on a catalytic subunit-Sepharose column. The affinity purified antibody was equally effective in precipitating purified catalytic subunit prepared either from bovine skeletal muscle (type I) or bovine cardiac muscle (type II) protein kinases (data not shown). The anticatalytic subunit antibody also precipitated catalytic subunit from reconstituted type I and type II holoenzyme. In the case of type I holoenzyme, immunoprecipitates contained C-subunit but no R^I regulatory subunit. Since the type I holoenzyme preparation was purified away from free C-subunit before immunoprecipitation (Fig. 2A) and since it showed an 8.3-fold stimulation of enzyme activity by cAMP we do not believe the antibody bound free catalytic subunit. Rather we interpret these results to indicate that binding of antibody to C-subunit can displace R^I regulatory subunit. Similarly, the type II holoenzyme was also separated from unbound C-subunit (Fig. 2B). Whether the antibody can also displace R^{II} is less clear-cut since the presence or absence of R^{II} in the immunoprecipitate is obscured by IgG heavy chain. What seems clear from these studies is that the anti-C antibody can recognize and precipitate the C-subunit in both type I and type II holoenzymes.

The specificity of the antibody was tested by ^{125}I -antibody immunodetection and by immunoprecipitation of ^{35}S -labeled cell extracts. In CHO cells, catalytic subunit was the only protein immunospecifically precipitated by the antibody. By this criterion, no immunologically related proteins in extracts of CHO fibroblasts were found. Preadsorption of the antibody with purified catalytic subunit before immunoprecipitation or immunofluorescence led to the specific disappearance of the M_r 40,000 band and abolished the fluorescence in stained cells.

An independent line of evidence that indicates specific binding of the anticatalytic subunit antibody to the enzyme is the

observation by Fletcher and Byus (39) that the antibody inhibits the binding of fluorescein-conjugated protein kinase inhibitor to the enzyme in cultured cells.

Redistribution of Catalytic Subunit in CHO Cells Treated with cAMP

The immunofluorescent localization of catalytic subunit in CHO cells reported here shows that the majority of the enzyme is distributed diffusely throughout the cytoplasm with much less enzyme evident in the nucleus. Nuclei have previously been shown to contain little or no cAMP-dependent protein kinase activity using biochemical methods. Dissociation of the holoenzyme in CHO cells by 8-Br-cAMP does not result in a redistribution of catalytic subunit. No change in the pattern or intensity of fluorescence was apparent. This observation is in contrast to the situation in rat liver in which glucagon treatment has been shown to increase catalytic subunit fluorescence in the cytoplasm and nucleus of rat liver parenchyma cells (40). We have obtained preliminary results showing a redistribution and immunospecific localization of catalytic subunit in the nucleus in regenerating rat liver. It will be of interest to determine if *in vitro* hormonal stimulation of cultured fibroblasts will elicit a similar redistribution of the catalytic subunit.

The fluorescence concentrated in ruffling membrane of Swiss 3T3 and WI-38 cells may reflect areas of thick cytoplasm. If, however, these regions are not especially thickened when compared to other areas along the cell periphery, then catalytic subunit may be localized in ruffles where it could participate in particle uptake processes and in membrane reorganization. An involvement of cAMP-dependent protein kinase in these functions is suggested by the finding of cAMP at sites of

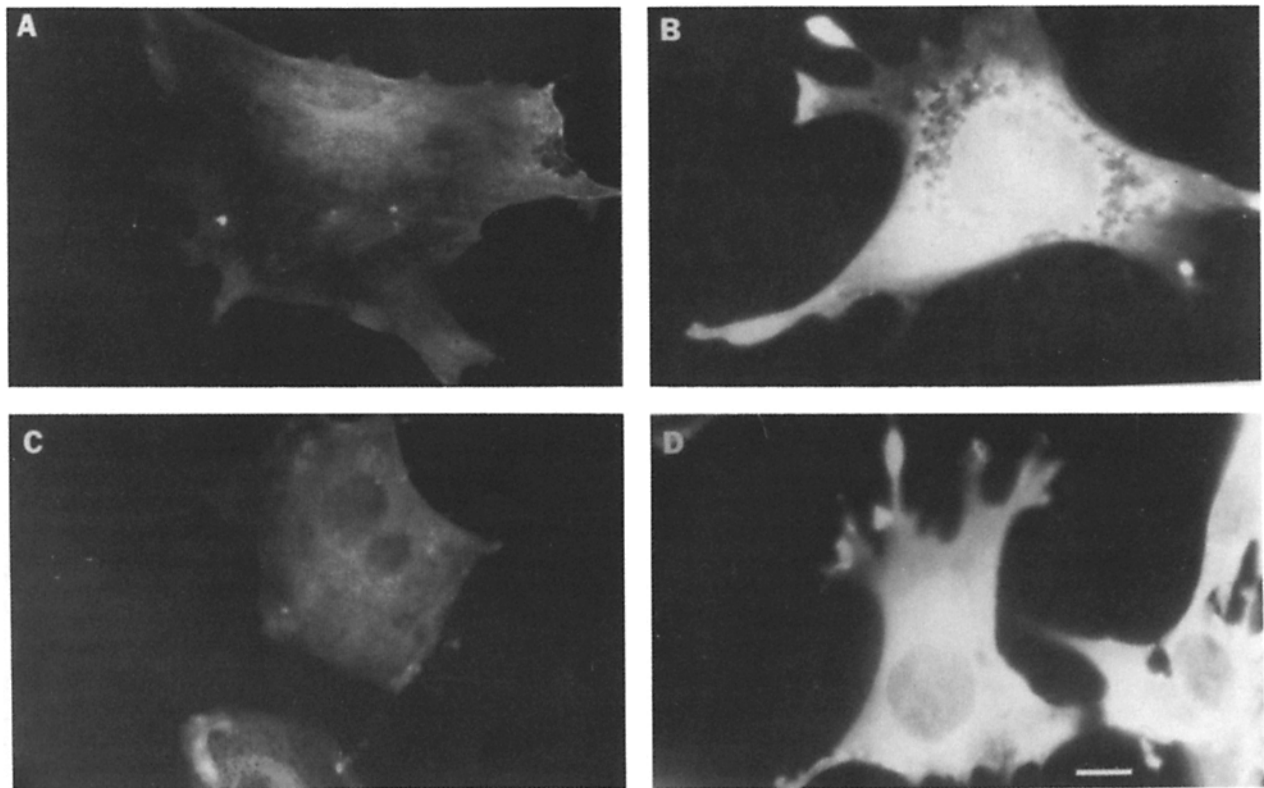


FIGURE 7 Immunofluorescent staining for catalytic subunit in cultured fibroblasts. Bovine endothelial tracheas (A), Swiss 3T3 (B), normal rat kidney (C), and WI-38 human lung (D) cells were fixed, permeabilized, and stained with 30 μ g anticatalytic subunit antibody and rhodamine-rabbit-anti-goat IgG. Bar, 10 μ m.

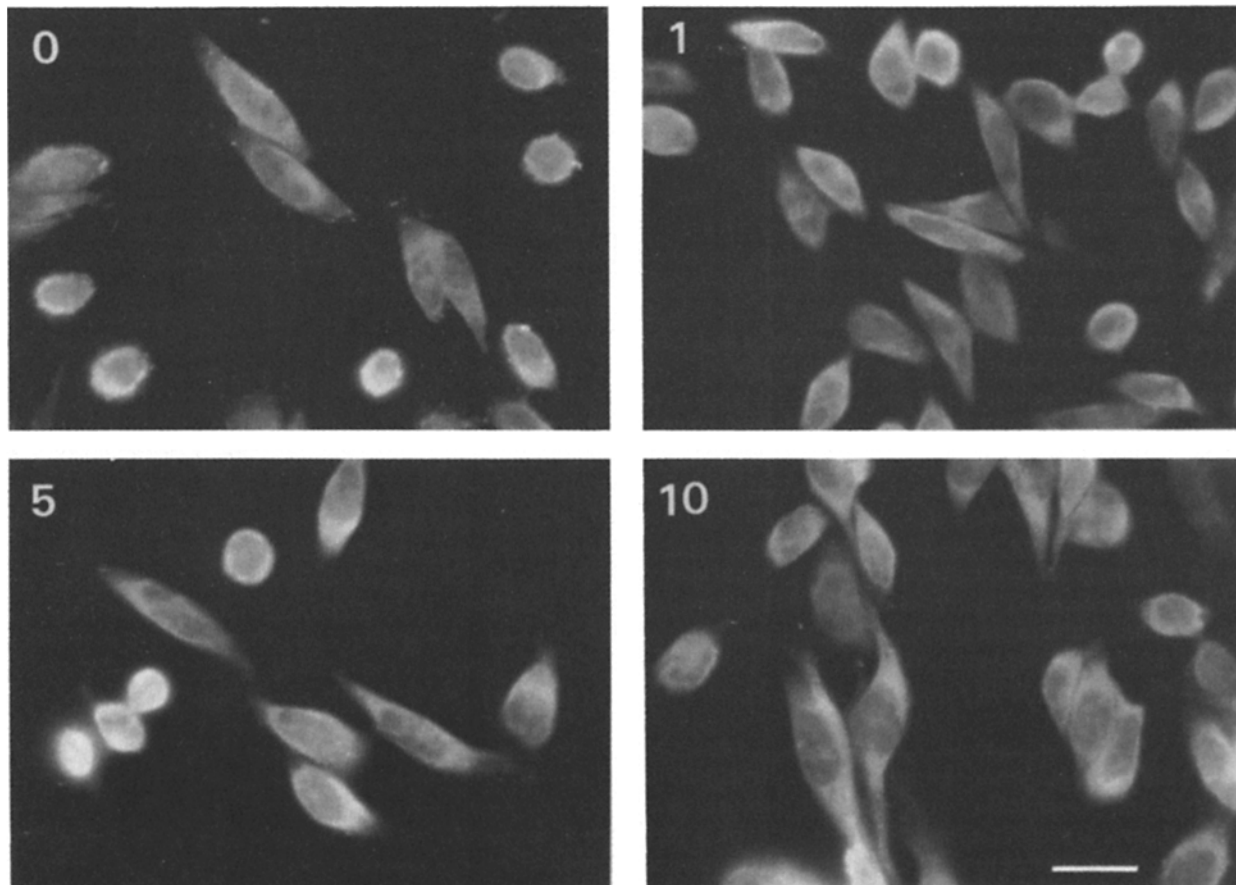


FIGURE 8 Catalytic subunit immunofluorescence in CHO cells treated with 8-bromo-cAMP. Cells were treated for 0–10 min as indicated with 1 mM 8-bromo-cAMP and then fixed and stained with anti-C antibody. Bar, 15 μ m.

phagocytosis in polymorphonuclear leukocytes (12).

These studies show that antibodies specific for the catalytic subunit of type I or type II cAMP-dependent protein kinases can be raised in goat. These antibodies localize catalytic subunit in cultured cells by indirect immunofluorescence and provide evidence that in cultured cells catalytic subunit is predominantly distributed in the cytoplasm. Dissociation of holoenzyme by cAMP does not lead to redistribution of catalytic subunit in CHO cells.

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