A Rapid Procedure for Preparing Fluorescein-labeled Specific Antibodies from Whole Antiserum: Its Use in Analyzing Cytoskeletal Architecture

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ABSTRACT A rapid method for the direct conjugation of affinity-purified antibodies with fluorescein (termed DCAPA) is described. This procedure involves the immobilization of antibodies as antigen-antibody complexes on nitrocellulose blots, and subsequently the bound antibodies are reacted with fluorescein isothiocyanate. An enriched sample of smooth muscle tropomysin transferred to nitrocellulose paper by the Western blotting procedure has been used as the affinity medium for purification of specific tropomyosin antibody from whole rabbit antiserum. Direct conjugation of the antibody with fluorescein was carried out following the binding of antibody to antigen. Direct conjugation and affinity purification of antibodies directed against tropomyosin was accomplished in 2–3 d using an enriched tropomyosin sample and whole antiserum directed against tropomyosin. The immunofluorescence images obtained with this procedure exhibit distinct advantages with regard to background fluorescence and overall specificity of antibody binding. The usefulness of this direct conjugation method in various experimental protocols is discussed.

Immunofluorescence methods have been used to localize specific antigens at the cellular and subcellular levels for a number of years. The technique, devised by Coons (1), has played a major role in generating new insights into cell structure-function relationships of a host of antigens. Undoubtedly, immunofluorescence will continue to play a pivotal role in cell biology due to the recent introduction and widespread use of monoclonal antibodies.

The majority of studies employing immunofluorescence microscopy utilize the indirect method (1). This method involves reacting fixed-permeabilized cells or tissues with an antibody preparation (whole antiserum, IgG fraction, specific IgGs, or monoclonal antibody), followed by another fluorochrome-labeled antibody preparation that reacts with all, for example, IgG molecules from a given species. Thus, if rabbit antiserum is used as the primary antibody, then goat anti-rabbit IgG conjugated with fluorescein usually represents the secondary antibody solution. In this fashion, regions of the cell or tissue containing the first antibody fluoresce when observed with a fluorescence optical system. There are considerable drawbacks to this relatively simple indirect procedure which include frequent problems with high background fluorescence probably due to nonspecific binding of antibodies, especially in those observations employing whole antiserum.

Fujiwara and Pollard (2) have elegantly demonstrated the advantages of procedures involving the use of directly conjugated, affinity-purified antibodies for fluorescence microscopy. Significant improvements in specificity of fluorescent staining reactions and microscopic resolution are evident with these preparations. The procedure of Fujiwara and Pollard (2) involves the separation of the IgG fraction from antiserum followed by reaction with fluorescein isothiocyanate (FITC[1]) or tetramethyl rhodamine isothiocyanate. Unconjugated fluorochrome is removed with a G-25 Sephadex column, followed by DEAE-cellulose chromatography to remove over and undercoupled IgG. The fluorochrome-conjugated IgG is then purified by affinity chromatography. The procedure requires numerous chromatographic steps, which are both time consuming and expensive. In addition, there is a requirement for significant amounts of highly purified antigen in the affinity chromatography steps. In many cases, this latter requirement may be extremely difficult or even impossible to achieve, as many antigenic proteins are minor constituents of cells or tissues.

A new procedure (3), utilizing electrophoretic methods, has

[1] *Abbreviations used in this paper:* DCAPA, direct conjugation of affinity-purified antibodies; FITC, fluorescein isothiocyanate.

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eliminated most of the steps in purification of antibodies and antigens as a prerequisite for affinity purification of specific IgGs. In this procedure, the desired antigen is separated from contaminants by SDS-PAGE and then transferred to diazophenylthioether paper by the Western blotting procedure (4). Antiserum is incubated with the western blot, and specific IgGs are then eluted from the portion of the blot containing the protein of interest. In this paper, we describe the use of this procedure in obtaining directly conjugated affinity-purified antibodies (DCAPA) by incorporating fluorochrome coupling steps while specific antibodies are bound to the paper during the affinity purification process.

MATERIALS AND METHODS

Cell Culture: Chicken embryo fibroblasts and human fibroblasts (ENSON, American Type Culture Collection) were grown in Dulbecco's Modified Eagle's medium (H-21) containing 10% fetal calf serum (Gibco Laboratories, Grand Island, NY), 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were cultured in 100-mm tissue culture dishes and subcultured by treatment with 0.05% trypsin-EDTA (Gibco Laboratories).

Preparation of Antigen: Smooth muscle tropomyosin was prepared according to the method of Hartshorne and Mueller (5). Tubulin was prepared according to the Shelanski procedure (6). Microtubule-associated proteins were removed by phosphocellulose chromatography as described by Weingarten (7). Tubulin was then modified with glutaraldehyde (8) before injection.

Preparation of Antibody: Preimmune serum was collected from New Zealand white rabbits (~10 ml/rabbit) and screened by indirect immunofluorescence (see below) for autoimmune antibodies directed against cytoskeletal proteins. Selected rabbits were injected subcutaneously in the foot pads and at several sites along their dorsal surface with 0.25–1.0 mg of antigen in complete Freund's adjuvant (1:1, vol/vol). 3 wk later, each rabbit was boosted with 0.5–1.0 mg of antigen in incomplete Freund's adjuvant at several sites along the dorsal surface of the animal. Blood was taken from the rabbits' ear vein 1 wk following the final injection, using an ear-bleeding apparatus (Bellco Glass, Inc., Vineland, NJ). The immune serum was collected following centrifugation of clotted blood for 20 min at 5,000 *g* in a Sorvall RC-5B (SS-34 rotor, DuPont de Nemours, E.I. & Co., Inc./Sorvall Instruments Div., Newtown, CT). Rabbits producing desired antibodies were boosted and bled every 2 wk.

Electrophoretic Procedures: SDS PAGE was carried out according to the Laemmli procedure (9) using either 7.5 or 10% polyacrylamide. Electrophoretic transfer of proteins from these gels to nitrocellulose paper (0.22-μm pore size Schleicher & Schuell, Inc., Keene, NH) was carried out in either a Hoefer Transphor (Hoefer Scientific Instruments, San Francisco, CA) or E-C Transfer Apparatus according to the techniques of Towbin et al. (4). Following SDS PAGE, gels were rinsed in transfer buffer containing 25 mM Tris and 192 mM glycine in 20% methanol and placed in the transfer apparatus overlayed with a sheet of nitrocellulose paper. This preparation was sandwiched between pieces of filter paper (Whatman Chemical Separation, Inc., Clifton, NJ). Electrophoretic transfer was carried out at 0.1–0.2 A for 10–16 h. Following removal from the transfer apparatus, polyacrylamide gels were stained with Coomassie Brillant Blue R. This permitted the determination of which bands did and which bands did not transfer to the nitrocellulose paper. The nitrocellulose sheet was divided and treated as outlined in Fig. 1. Vertical strips (*A* and *B*) were sliced from the sides of the nitrocellulose sheet and stained with 0.1% amido black, 5% MeOH, and 10% acetic acid for ~5 min and destained in 5% MeOH, 10% acetic acid. This permitted the visualization of the bands of protein that were transferred to the paper from the gel. The remainder of the nitrocellulose sheet was incubated for ~1 h in blocking buffer (10% fetal calf serum, 2.5% BSA in Dulbecco's phosphate-buffered saline [PBS: 0.17 M NaCl, 3.0 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4]).

Antibody Adsorption and Localization on Protein Transfers: The "blocked" nitrocellulose transfer was rinsed several times in PBS for ~15 min and then divided in the following manner (see Fig. 1): Two vertical strips (*C* and *D*) and one horizontal strip (*E*) (locating a particular protein by aligning the amido black stained strips [*A* and *B*] with the remaining sheet of nitrocellulose) were cut from the transfer. One of the vertical strips (*D*) was stored for reblotting with purified antibody (see below), while strips *C* and *E* were incubated with appropriately diluted antiserum (1:20 to 1:200) for ~2–16 h. These strips were rinsed in several changes of PBS for 15–30 min and then strip C was developed in a PBS solution containing 0.05% 4 chloronaphthol and 0.01% H_2O_2. A purple-black reaction product was observed wherever the antibody was bound.

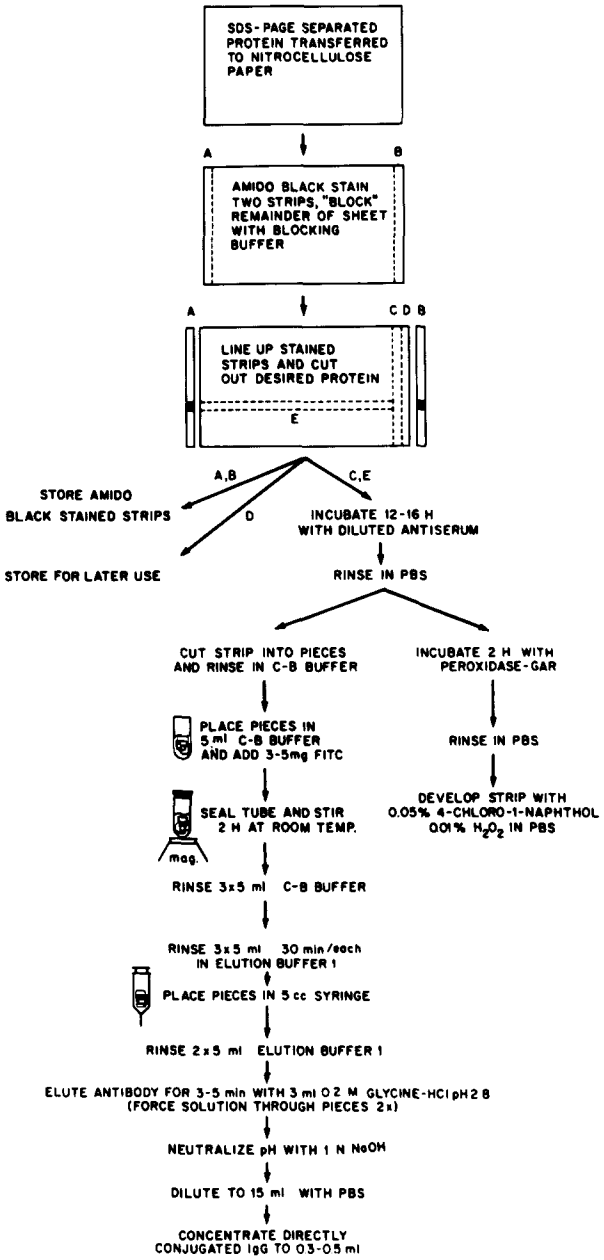

FIGURE 1 A flow diagram showing the protocol for the preparation of DCAPA. *C-B buffer*, carbonate-bicarbonate buffer; *FITC*, fluorescein isothiocyanate; *Peroxidase GAR*, peroxidase labeled goat anti-rabbit IgG.

FITC Conjugation of Antibodies Immobilized on Nitrocellulose: The horizontal strip (*E*) containing the antigen-antibody complex was rinsed with several changes of 0.5 M carbonate-bicarbonate (*C-B*) buffer (3.7 g $NaHCO_3$, 0.6 g Na_2CO_3 to 100 ml, pH 8.8–9.0) (10). Strip E was cut into short pieces and placed in 3–5 mg of FITC (Isomer I, Sigma Chemical Co., St. Louis MO) dissolved in 5 ml of CB buffer in a 15-ml corex tube (Fig. 1). 2 ml of C-B buffer was then used to rinse the FITC powder from the side of the tube which was then stoppered, completely covered in foil, and placed on a magnetic stirrer for ~2 h at room temperature. The strips were then rinsed with several changes of C-B buffer.

Elution of FITC-conjugated Antibodies: FITC-conjugated antibodies were eluted from the nitrocellulose strips (*E*) according to the method described by Olmsted (3). All solutions were maintained at 4°C. The strips were washed with several changes of elution buffer 1 (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 150 mM NaCl) and placed in a 5-cc syringe. Two 5 ml aliquots of elution buffer 1 were forced through the strips. 3 ml of 0.2 M glycine-HCl, pH 2.8 was added to the syringe and left for 2–5 min. This solution

was forced through the paper twice and then neutralized with 1 N NaOH. The sample was diluted to a final volume of 10–15 ml with PBS and concentrated to 0.2–0.5 ml with a Millipore CX-10 ultrafiltration kit (Millipore Corp., Bedford, MA). 25 μl of the concentrated sample was diluted in 1–2 ml of PBS and incubated with the remaining vertical strip (*D*) to test the specificity and reactivity of the eluted material.

Preparation of Cells for Immunofluorescence Microscopy: Cells were grown on 22-mm²-glass coverslips that had been placed in 35 mm tissue culture dishes (Corning Glass Works, Corning, NY). The cells were rinsed with three changes of PBS and fixed and permeabilized by either method A: 5 min in 3.7% formaldehyde, rinsed with three changes of PBS, and placed in cold acetone for 1 min; (or method B:) direct immersion in cold acetone (−20°C) for 1 min. All coverslips were air-dried following removal from the acetone and then overlaid with 50 μl of antibody solution in a humidified chamber at 37°C for 30 min. Coverslips were then rinsed with distilled water and mounted on glass slides in Gelvatol (Monsanto Co., St. Louis, MO) (11).

Double-label preparations were fixed and permeabilized by method A before incubation with rabbit antiserum directed against tubulin. After rinsing in PBS, the coverslips were incubated with rhodamine-conjugated goat anti-rabbit IgG (Miles Laboratories, Inc., Elkhart, IN), rinsed in PBS, and fixed again in 3.7% formaldehyde for 5 min. This latter step assures that the rhodamine-conjugated goat anti-rabbit IgG will not become redistributed and contribute to background fluorescence (H. Y. Yang, Northwestern University Medical School, personal communication). The coverslips were then reincubated with antitubulin serum to block any free and active rabbit IgG-binding sites before final incubation with the fluorescein-conjugated tropomyosin antibody obtained by the direct conjugation of affinity-purified antibodies (DCAPA)-procedure. The coverslips were washed, mounted in Gelvatol, and observed as described below.

Slides were viewed with a Zeiss Photomicroscope III (PM III) fitted with a III-RS epi-illuminator containing fluorescein and rhodamine filters. A 75-W Xenon lamp (Carl Zeiss, Inc., Thornwood, NY) was used for fluorescein fluorescence and a 100-W Hg lamp was used for rhodamine fluorescence. Photographs were taken on Kodak Plus-X film and developed in Diafine (Acufine, Inc., Chicago, IL) two-stage developer.

RESULTS

DCAPA

DCAPA were eluted from nitrocellulose transfers of protein preparations enriched for smooth muscle tropomyosin (see Materials and Methods). Fig. 2, lane *A* shows the enriched tropomyosin sample transferred to nitrocellulose paper and stained with amido black. The typical tropomyosin gel profile containing two major proteins of molecular weights 36,000 and 39,000 are clearly seen as well as some minor contaminants of lesser molecular weights. The tropomyosin bands stained with amido black are easily aligned with those detected with peroxidase-labeled goat anti-rabbit IgG following the Towbin procedure and incubated in rabbit antitropomyosin serum (see Materials and Methods) (Fig. 2, lane *B*). The reactivity of diluted whole serum directed against tropomyosin (Fig. 2, lane *B*) and the specificity of reblotted DCAPA from the same whole antiserum (Fig. 2, lane *C*) are readily apparent. The decrease in background staining of the Western blots in the DCAPA preparations is obvious and is typical of the reblotted samples of directly conjugated antibody preparations (compare Fig. 2, *B* and *C*).

Immunofluorescence Microscopy

The specific advantage of using DCAPA relative to other antibody preparations is especially evident when comparing direct and indirect immunofluorescence images. Staining with whole serum directed against smooth muscle tropomyosin using the indirect method usually results in a stress fiber pattern with a significant amount of background cytoplasmic and occasionally nuclear fluorescence (Fig. 3*A*). At least some of this background fluorescence may have been due to the nonspecific binding of fluorescein-labeled goat anti-rabbit IgG

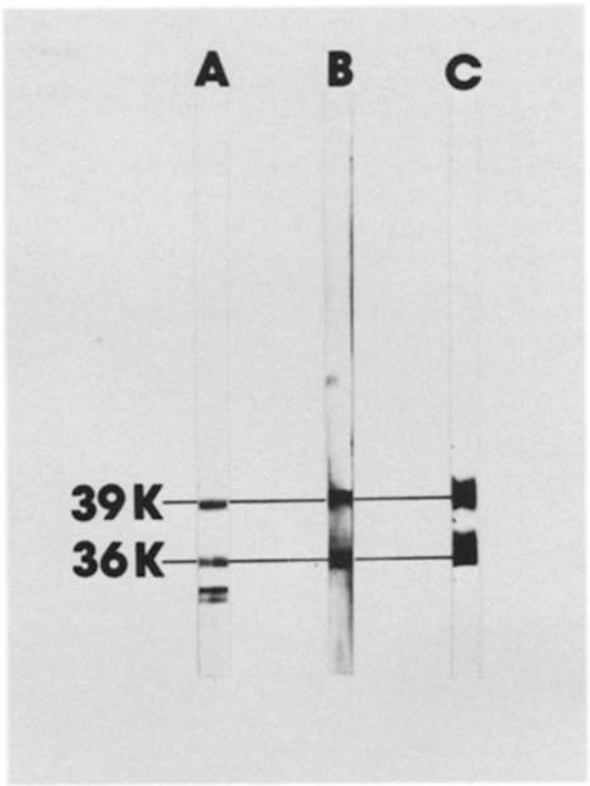

FIGURE 2 Western blots of smooth muscle tropomyosin. (*A*) Amido Black stain of tropomyosin preparation; (*B*) smooth muscle tropomyosin antiserum (diluted 1:20 in PBS) staining detected with peroxidase labeled goat anti-rabbit IgG; (*C*) DCAPA directed against smoth muscle tropomyosin stained as in *B*. *K*, × 10^3.

(Fig. 3*B*). Direct coupling with fluorescein and affinity purification of the antibody (the DCAPA method) eliminated, in the majority of cells, most of the cytoplasmic background and nuclear fluorescence (Fig. 3*C*).

DCAPA have also been used in double-fluorescence labeling experiments (see Materials and Methods). Fig. 4*A* shows the indirect staining pattern obtained with tubulin antibody and rhodamine-conjugated goat antirabbit IgG. The staining pattern obtained with DCAPA directed against tropomyosin in the same cell is shown in Fig. 4*B*. The two distinct staining patterns are apparent, with no superimposition of the fluorescein and rhodamine images.

DISCUSSION

Immunofluorescence microscopy has been used to localize antigens within cells for many years. Improvements in fixation and antibody preparation techniques have been important in advancing the reproducibility and resolution of this form of light microscopy. The development of procedures for the direct conjugation of antibodies with fluorochromes and their subsequent purification by affinity chromatography (see e.g., reference 2), as well as the use of monoclonal antibodies, have enhanced both the specificity and resolution of the immunofluorescence technique. The method described in this paper for the fluorescein conjugation and affinity purification of antibodies has numerous advantages over more conventional procedures. One of the most important of these is that the time involved in obtaining a useful end product of directly conjugated specific antibody was greatly reduced. This is due

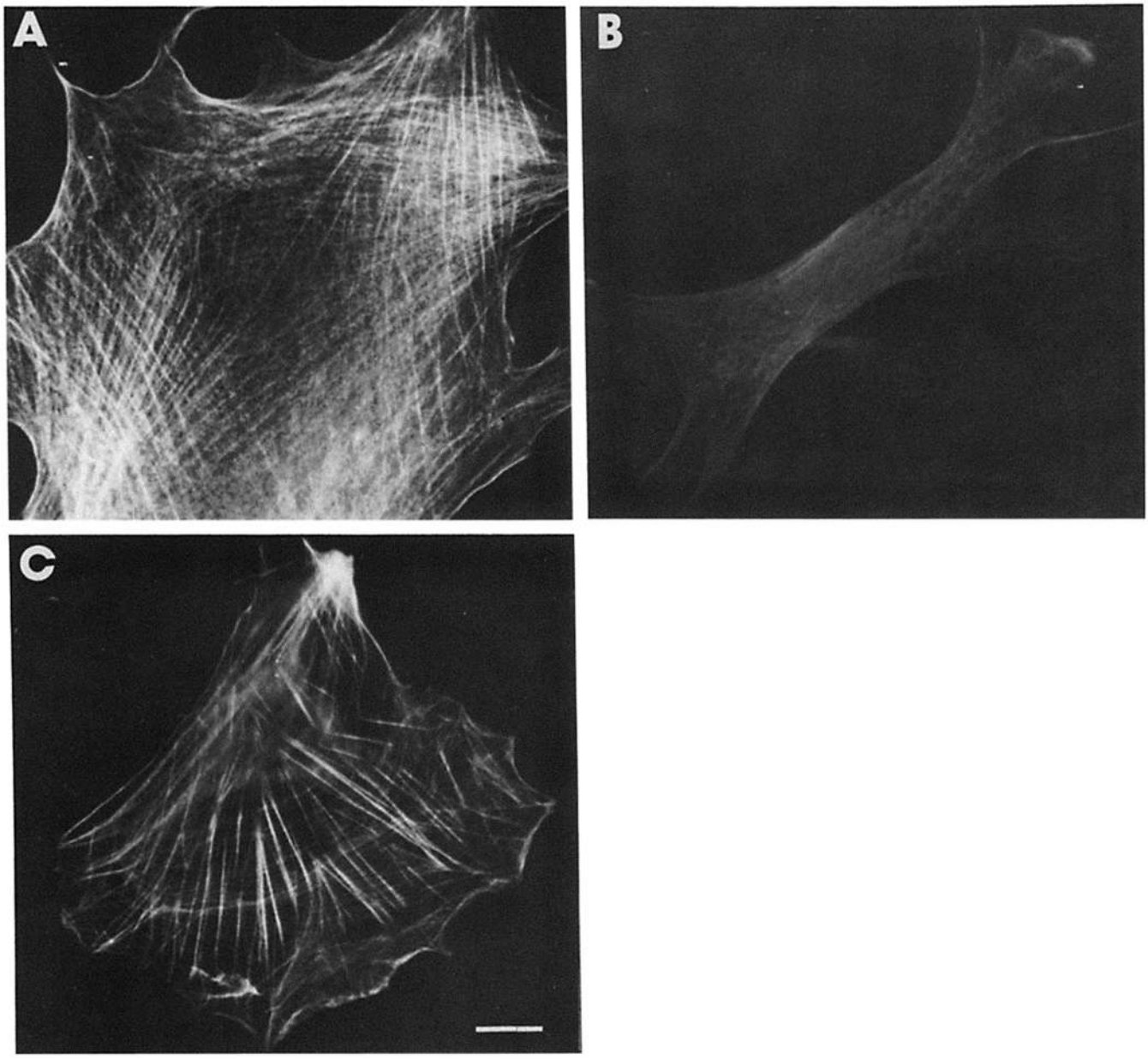

FIGURE 3 Immunofluorescence observations of chick embryo fibroblasts stained with smooth muscle tropomyosin antibody preparations. (*A*) Indirect method. Smooth muscle tropomyosin antiserum (diluted 1:20 in PBS) detected with fluorescein-labeled goat anti-rabbit IgG. A stress fiber-staining pattern is evident, along with "background" fluorescence. (*B*) Fluorescein-labeled goat anti-rabbit IgG control showing background staining level with this antibody preparation; (*C*) directly conjugated affinity-purified antibody directed against smooth muscle tropomyosin. In most cells, "background" fluorescence is reduced and stress fibers are more apparent; all cells fixed by method B. Bar, 100 μm. × 1,000.

to the fact that the DCAPA method obviates many of the steps needed to fractionate IgG from whole antiserum and eliminates the numerous chromatographic steps required to separate fluorescein-labeled IgG from unbound FITC as well as over- and undercoupled IgGs (2).

It follows that another important advantage of the procedure is that whole complex serum containing any number of antibodies against different proteins could be adsorbed to the nitrocellulose blots. No other manipulations of the serum obtained from the immunized animal were necessary. Crude or partially enriched preparations of antigen could be used in both the immunization regimen and the DCAPA method. Although we have not yet tried the DCAPA method on a large number of different antibodies, both tubulin and α-actinin antibodies have also been successfully conjugated and purified by this procedure (unpublished results).

The major limiting factor of the DCAPA method is that polypeptide antigens must be separable and easily identified by one-dimensional, or (if greater resolution is required) two-dimensional gel electrophoresis. In addition, the proteins must be capable of being transferred to nitrocellulose with reasonable efficiency, and subsequently they must retain their ability to bind specifically to antibodies, which in turn must be separable from the bound antigen in a fashion that retains

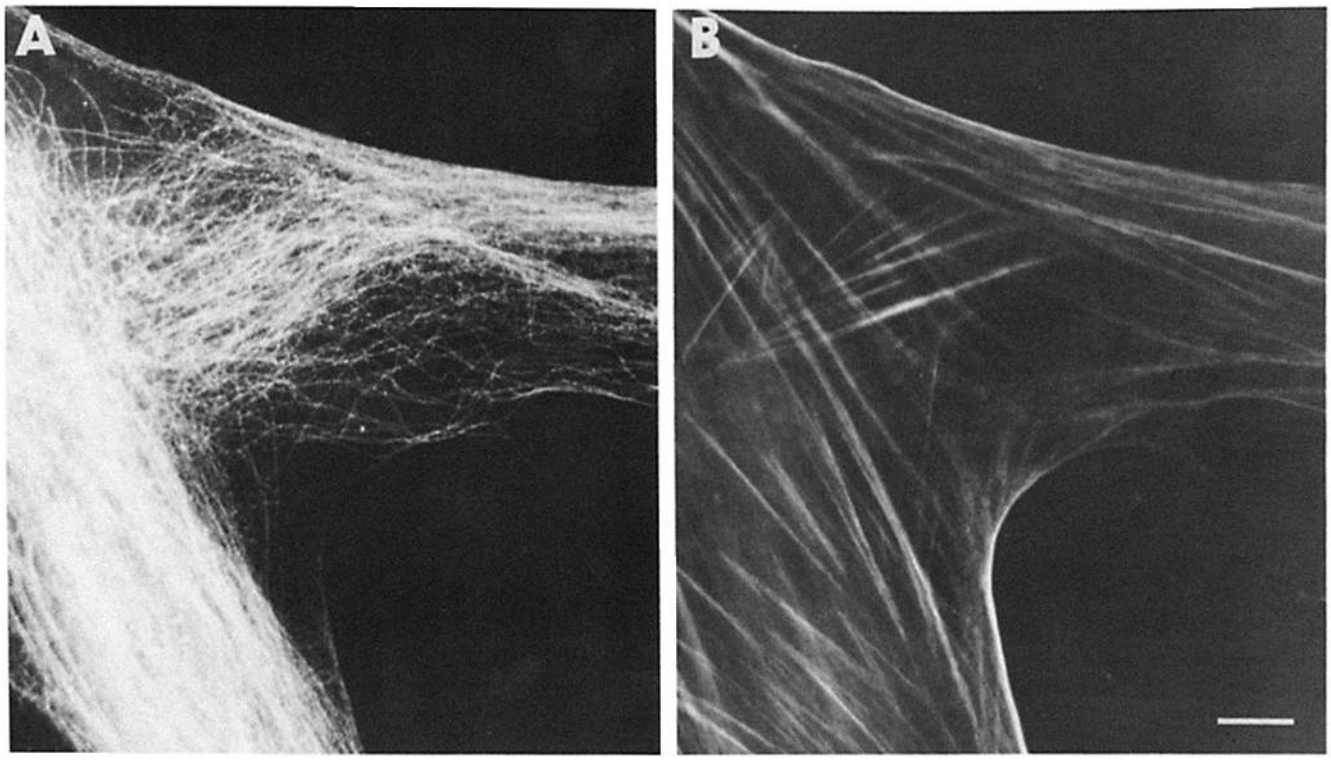

FIGURE 4 Double-label immunofluorescence observation of ENSON cells with tubulin antiserum detected with rhodamine-labeled goat anti-rabbit IgG and DCAPA directed against tropomyosin (labeled with fluorescein). *A* exhibits the characteristic microtubule pattern of ENSON cells. *B* shows the stress fiber image obtained with directly conjugated tropomyosin antibody in the same cell. Overall background fluorescence is significantly decreased relative to that seen using double indirect immunofluorescence with rabbit antibodies. No superimposition of the two distinct images is evident, even though both the tubulin and tropomyosin antibodies were produced in rabbits; cells fixed by method A. Bar, 100 μm. × 1,000.

antibody activity. Thus, antibodies directed against antigenic determinants of the conformational or structural type could probably not be used in this procedure. Antigens of this type are sensitive to the denaturation steps used in SDS PAGE and they subsequently should not bind antibody (12). Finally, it should be mentioned that the DCAPA method is primarily of use in cases in which only small amounts of antigen are available in pure form and in which small amounts of directly conjugated antibodies are needed (e.g., immunolocalization at the light and electron microscope levels of resolution).

The original procedure for affinity purification of antibodies utilized diazotized papers as opposed to the nitrocellulose paper described here (3). The smooth muscle tropomyosin used in these experiments transferred and adsorbed efficiently to the nitrocellulose sheets. This preparation of protein does not appear to require the covalent linkages afforded by diazophenylthioether paper. Other antigens may not be so tightly bound to the nitrocellulose and may require the covalent binding capacity of diazophenylthioether paper to stabilize them on the blots.

The buffer system for FITC conjugation, as well as the incubation times and conditions, have been selected for the rabbit tropomyosin antiserum used in this study. Usable FITC IgG conjugates were obtained in 2 h at room temperature (22°C) at pH 8.8–9.0. Several protocols for FITC conjugation that used longer incubation times (18–24 h) in the cold (4°C) or neutral pH buffers ($NaPO_4$, pH 7.4) may be optimal for other antigen-antibody preparations. Many of these variations of the protocol could be tested on a single preparation by dividing the antigen containing horizontal strips (*E*, Fig. 1) into several pieces prior to the FITC conjugation step. Following release of fluorescein-labeled IgG, the resulting samples should be concentrated to 25–50 μl before testing to maximize the concentration (i.e., reactivity) of the conjugated antibodies. Another advantage of the DCAPA method is that any minor fraction of "overcoupled" IgG, which loses its antigen binding activity, and contributes to nonspecific binding and background fluorescence (2), will most likely be excluded during the rinsing process following the coupling reaction. We believe that this was the case as the ability of overcoupled IgGs to become reassociated with antigen molecules in the nitrocellulose strips should be greatly diminished (13). We also believe that it is unlikely that a significant amount of antibody was lost due to overcoupling (e.g., fluorescein conjugation in the antigen binding site) as the majority of the antigen-binding sites of IgG molecules should be protected during the FITC reaction. This was due to the fact that antigen-binding sites are specifically bound to antigenic determinants during the coupling reaction.

The separation of the antigen-antibody complex was accomplished by a pH shock procedure (2). The antigen-containing nitrocellulose strips, as with most affinity chromatography resins, can be used several times following antibody release. The diazophenylthioether paper strips are more suitable for extended use than the noncovalently linked antigen-nitrocellulose strips, due to slow diffusion of antigen from the latter strips. Antigen-containing strips should be stored in 0.02% sodium azide at 4°C and protected from light. It should

also be pointed out that many antibodies with high antigen affinity cannot be eluted at low pH. In these cases other techniques could be attempted, such as high salt elution. It is likely, however, at least with polyclonal antibody preparations, that some of the antibodies directed against a specific antigen will be eluted by the pH method.

DCAPA should be useful for microinjection experiments. Most of these experiments utilized fluorescently labeled purified proteins (see, for example, references 14 and 15) or unlabeled affinity-purified antibodies (see, for example, reference 16). The latter experiments required fixation, permeabilization, and staining with a fluorochrome-labeled second antibody to localize the injected antibodies. DCAPA can be injected and observed in a living cell so that the fate of the injected antibody could be followed directly (using low-light sensitive TV systems) without further perturbation of the cells with fixatives and staining solutions.

In summary, the DCAPA procedure allows the separation of specific antibodies from complex antisera and relatively crude protein preparations. This method should provide fluorochrome-labeled specific antibodies for a wide variety of cell biological experiments. It is potentially very useful where small amounts of antigen of known and unknown function are only detectable by one- or two-dimensional gel electrophoresis and Western blotting (4).

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