A Smooth Muscle-specific Monoclonal Antibody Recognizes Smooth Muscle Actin Isozymes

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ABSTRACT Injection of chicken gizzard actin into BALB/c mice resulted in the isolation of a smooth muscle-specific monoclonal antibody designated CGA7. When assayed on methanol-Carnoy's fixed, paraffin-embedded tissue, it bound to smooth muscle cells and myoepithelial cells, but failed to decorate striated muscle, endothelium, connective tissue, epithelium, or nerve. CGA7 recognized microfilament bundles in early passage cultures of rat aortic smooth muscle cells and human leiomyosarcoma cells but did not react with human fibroblasts. In Western blot experiments, CGA7 detected actin from chicken gizzard and monkey ileum, but not skeletal muscle or fibroblast actin. Immunoblots performed on two-dimensional gels demonstrated that CGA7 recognizes γ-actin from chicken gizzard and α- and γ-actin from rat colon muscularis. This antibody was an excellent tissue-specific smooth muscle marker.

Antibodies to intermediate filament proteins are useful tissue-specific markers because the protein composition of these structures markedly differs, depending on cell type (1). Other cytoskeletal components exhibit more restricted tissue specificities. For example, different tissues contain actin isozymes that vary in amino acid sequence and isoelectric point (α-, β-, and γ-mobilities) (2–5). Skeletal, cardiac, and vascular smooth muscle each contain unique α actins (6–8). Nonmuscle cells contain actins of β- and γ-mobility and chicken gizzard smooth muscle contains a γ-actin that differs from the nonmuscle isozyme (9). These six different actin isozymes share >90% sequence homology throughout the entire molecule, but each has a unique sequence in the first 18 residues at the amino terminus (6–10).

It has been difficult to isolate isozyme-specific antiactin antibodies because of this high degree of homology, but polyclonal, isozyme-specific antibodies have been described (11, 12). Recently, investigators have isolated isozyme-specific sera by making antibodies to the amino terminus of skeletal muscle α-actin (13) or by selective absorption of a polyclonal antisera with skeletal, smooth, or nonmuscle actin (14–16). Additionally, a monoclonal antiactin has been generated that reacts with only muscle actins (both smooth and striated) (15). We report here the isolation of a smooth muscle-specific monoclonal antibody that appears to selectively recognize smooth muscle actin species.

MATERIALS AND METHODS

Actin Purification and Isolation of Monoclonal Antibodies: Monoclonal antibodies to chicken gizzard actin preparations were raised according to the basic scheme previously outlined (17). Partially purified chicken gizzard actin, provided by Dr. Giulio Gabbiani (University of Geneva), was prepared according to the method of Hubbard and Lazarides (18). A series of four intraperitoneal injections of 100 μg of material suspended in phosphate-buffered saline (PBS) with extensive sonication was given to a BALB/c mouse over an 8-wk period. The spleen was then removed and a fusion procedure was performed with NS-1 cells as previously described (17).

Hybridoma supernatants were screened on acetone-fixed, frozen sections of rat intestine snap-frozen in isopentane which was cooled in liquid nitrogen. Undiluted supernatants were incubated on sections for 30 min at room temperature. After a brief wash in PBS, sections were incubated with fluorescein-conjugated goat anti-mouse IgM + IgG (Tago Inc., Burlingame, CA) for 30 min at room temperature. After a second wash in PBS, sections were overlaid with mounting medium (Aquamount) (Lerner Laboratories, New Haven, CT) and a glass coverslip. Screening was performed with a Zeiss Photoscope II (Carl Zeiss, Inc., Thornwood, NY) equipped for epifluorescence with appropriate excitation and emission filters.

Cells that produced supernatants positive on rat intestine muscularis were cloned and ascites fluid was isolated as previously described (17). One clone, designated CGA7, was isolated and characterized.

Actin Purification and Production of Rabbit Antiactin Serum: For production of the polyclonal sera, chicken gizzard actin was prepared according to the method of Herman and Pollard (19). Lyophilized actin powder (1.8 mg) was dissolved in reduced SDS sample buffer and then electrophoresed on a 3-mm Laemmli gel without urea (20). The actin band was visualized with 8-anilino-1-napthelene sulfonic acid; then it was cut from the gel, minced in a small amount of running buffer, and homogenized in a

Dounce glass homogenizer. The gel band mixture was emulsified thoroughly with an equal volume of Freund's complete adjuvant, and 7.0 ml of the antigen mixture was divided equally and injected subcutaneously into the back of two white New Zealand rabbits at six or seven sites each. After 4 wk, the rabbits were boosted with multiple subcutaneous injections of glutaraldehyde-treated actin (19) and a second boost was administered 2–3 wk later. Antisera were screened by double immunodiffusion (21).

Rabbit skeletal muscle actin was purchased from Sigma Chemical Co., St. Louis, MO.

Culture of Human Leiomyosarcoma: The leiomyosarcoma arose in the lower uterine segment of a 48-y-old woman. Tumor was minced with sterile scalpel blades and treated with 0.05% crude collagenase (Worthington Biochemical Corp., Freehold, NJ) in PBS for 20 min at 37°C. Material was centrifuged, and tissue chunks and single cells were plated onto tissue culture dishes coated with 0.67% gelatin. The cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum and penicillin-streptomycin. Primary cultures were transferred to Teflon-coated slides (Meloy Laboratories, Inc., Springfield, VA) for immunofluorescence studies.

Tissue Culture of Rat Aortic Smooth Muscle Cells: Aortic medial cells were prepared from 5-mo-old Sprague-Dawley male rats (Tyler Laboratories, Bellevue, WA). The aortic smooth muscle preparation was obtained from three or four thoracic aorta segments. Each aorta was opened and the endothelium was scraped off with a Teflon spatula. After a 30-min incubation in Waymouth's culture medium with 0.1% collagenase (146 U/mg) (Worthington Biochemical Corp.) and 0.05% elastase (Type I, 32 U/mg) (Sigma Chemical Co.) the adventitia was dissected off with watch makers forceps, leaving the aortic media. In the above (but fresh) enzyme mixture, the tissue was finely minced and incubated in a shaker bath at 37°C for 2–4 h, with gentle pipetting at 1-h intervals to aid tissue dissociation (22). After adequate dispersion, enzymes were inactivated by adding bovine serum (20% final concentration), and the suspension was filtered through a 250-μm wire mesh to remove tissue debris and undigested fragments. The filtrate was centrifuged at 200 *g* for 7 min, and the cell pellet was resuspended in Waymouth's medium with 10% bovine serum and seeded at a density of $\sim 10^4$ cells/cm^2.

Extraction of Human Skeletal Muscle, Monkey Ileum Smooth Muscle, and Cultured Human Fibroblasts: Human gastrocnemius skeletal muscle was obtained from an above-the-knee amputation for synoviosarcoma. The uninvolved muscle was freed of connective tissue and 7 g minced with scissors and washed with 10 mM Tris, 140 mM NaCl (pH 7.6) containing 0.5 mM phenylmethylsulfonyl fluoride and 5 mM EDTA. The tissue was homogenized in 30 ml of 1% SDS, 50 mM Tris (pH 6.8), and then boiled for 10 min. Particulate material was removed by centrifugation and the supernatant was stored at −20°C.

Monkey ileum muscularis was extracted as above. The mucosa was mechanically removed before homogenization.

Confluent cultures of human fibroblasts in 10-cm dishes (~ 2–3×10^6 cells/dish) were washed in PBS and solubilized in 1% SDS, 50 mM Tris (pH 6.8) at a ratio of 0.4 ml/10-cm dish.

Extraction of Rat Colon Muscularis: 5-mo-old Sprague-Dawley male rats (Tyler Laboratories) were killed with ether, and a segment of colon was placed in chilled Waymouth's culture medium. The pericolic fat was removed and the mucosa was scraped off with a Teflon spatula. The remaining muscularis was homogenized in 0.05 M Tris, (pH 6.8) containing 1 mM phenylmethylsulfonyl fluoride (1 ml/1-g tissue). The homogenate was treated with DNAase I (40 μg/ml final concentration) (Sigma Chemical Co.) for 30 min at 4°C; SDS and β-mercaptoethanol were then added to a final concentration of 2% and 5%, respectively. The sample was incubated at 70°C for 30 min and particulate matter was removed by centrifugation. For two-dimensional gels, 0.21 g urea and 0.04 ml Nonidet P-40 was added to 0.2 ml of sample buffer and then 0.50 ml of two-dimensional gel lysis buffer (23) was added. This material was then applied to an isoelectric focusing gel (see below).

One-dimensional Western Blot Experiments: Purified chicken gizzard actin or rabbit skeletal muscle actin was solubilized in SDS sample buffer at a concentration of 1 mg/ml. Extracts of human skeletal muscle, monkey ileum, and human fibroblasts were diluted 1:1 with 2 × SDS sample buffer with dithiothreitol. Samples were separated on an 8% polyacrylamide gel (20) and transferred overnight onto nitrocellulose paper as previously described (17, 24). Nitrocellulose paper was incubated with diluted ascites fluid in 5% BSA and was then counterstained with a 1:400 dilution of peroxidase-conjugated rabbit anti-mouse IgG purchased from Dako Corp. (Santa Barbara, CA), and Accurate Chemical & Scientific Corp. (Westbury, NY). Proteins on the nitrocellulose paper were detected by staining with Amido black as previously described (17).

Two-dimensional Gel Electrophoresis: Two-dimensional gels were performed by the methods of O'Farrell (23, 25) with only minor modifications (3). For good separation of the actin isotypes, only the pH 5–7 Ampholine was used in making the isoelectric focusing gels, and the gels were 18 cm long. Approximately 20 μg of protein was loaded onto each isoelectric focusing gel. Isoelectric focusing was performed at 1,000 V for 18 h, and then increased to 1,600 V for an additional hour. The gels were incubated in SDS sample buffer for 2 h and then mounted onto 12% acrylamide gels (sealed with 1% agarose) for the second dimension. After completion, the gels were silver stained (26) or transblotted.

To determine the actin isotype(s) recognized by CGA7, we first incubated immunoblots with CGA7, and then with peroxidase-conjugated rabbit anti-mouse IgG. The results were recorded. The same immunoblot was subsequently incubated with the rabbit polyclonal actin antibody followed by peroxidase conjugate swine anti-rabbit IgG (Dako Corp.).This second actin antibody, by reacting with muscle and nonmuscle isotypes, allowed the determination of actin isotype(s) recognized by CGA7.

Immunofluorescence: Immunofluorescence was performed on cells maintained on Teflon-coated multiwell slides (Meloy Laboratories, Inc.) fixed in −20°C methanol for 5 min as previously described (17). CGA7 decorates only cells fixed in methanol. Acetone or paraformaldehyde fixation results in negative immunofluorescence.

Immunocytochemistry: Hybridoma ascites fluids were assayed on methanol-Carnoy's fixed, paraffin-embedded human tissue obtained from surgical material exactly as described (27). Hybridoma ascites fluids were diluted 1:500–1,000 before use.

RESULTS

One antibody, designated CGA7, was isolated in the initial screen on rat intestine. It was then tested on methanol-Carnoy's fixed, paraffin-embedded tissue and acetone-fixed, frozen sections. Only the methanol-Carnoy's data will be presented because the two fixation procedures yielded similar results.

Immunocytochemistry

CGA7 decorated only smooth muscle cells, reacting with the muscularis and muscularis mucosa of the gastro-intestinal tract, the uterine myometrium, medial layer of all blood vessels, and mesenchymal components of the prostate (Fig. 1, *a–d*). All other tissues, including the endothelial cells of blood vessels, were nonreactive (Fig. 1, *c* and *e*), with the single exception of myoepithelial cells of the salivary gland and other organs (Fig. 1*f*). Neither skeletal nor cardiac muscle reacted with the antibody, but the antibody did recognize smooth muscle cells in the blood vessels in these tissues (Fig. 1, *e* and *g*). In contrast to CGA7, the polyclonal rabbit serum made against chicken gizzard actin identified all types of muscle, including skeletal muscle (Fig. 1*h*). However, the polyclonal antibody reacted more strongly with smooth muscle (data not shown). Finally, a monoclonal antivimentin antibody (27) stains mesenchymal tissues in a fashion totally different than CGA7, decorating blood vessels and stromal fibroblasts, and weakly staining the smooth muscle cells in a leiomyoma (Fig. 1, *i* and *j*).

We performed indirect immunofluorescence on a number of different cell lines and cell strains to identify intracellular structures recognized by CGA7. The antibody stains early passage rat aortic medial cells and human leiomyosarcoma cells in an actin-like pattern (28), decorating linear stress-fibers running the entire length of the cells (Fig. 2, *a* and *b*). These are the only cultured cells in our laboratory that reacted with CGA7. Human fibroblasts were negative with these antibodies (Fig. 2*c*), as were human tumor cells, mouse 3T3 cells and late passage monkey aortic smooth muscle cells (data not shown). As the rat aortic medial cells and human leiomyosarcoma cells were passaged in culture, they lost the

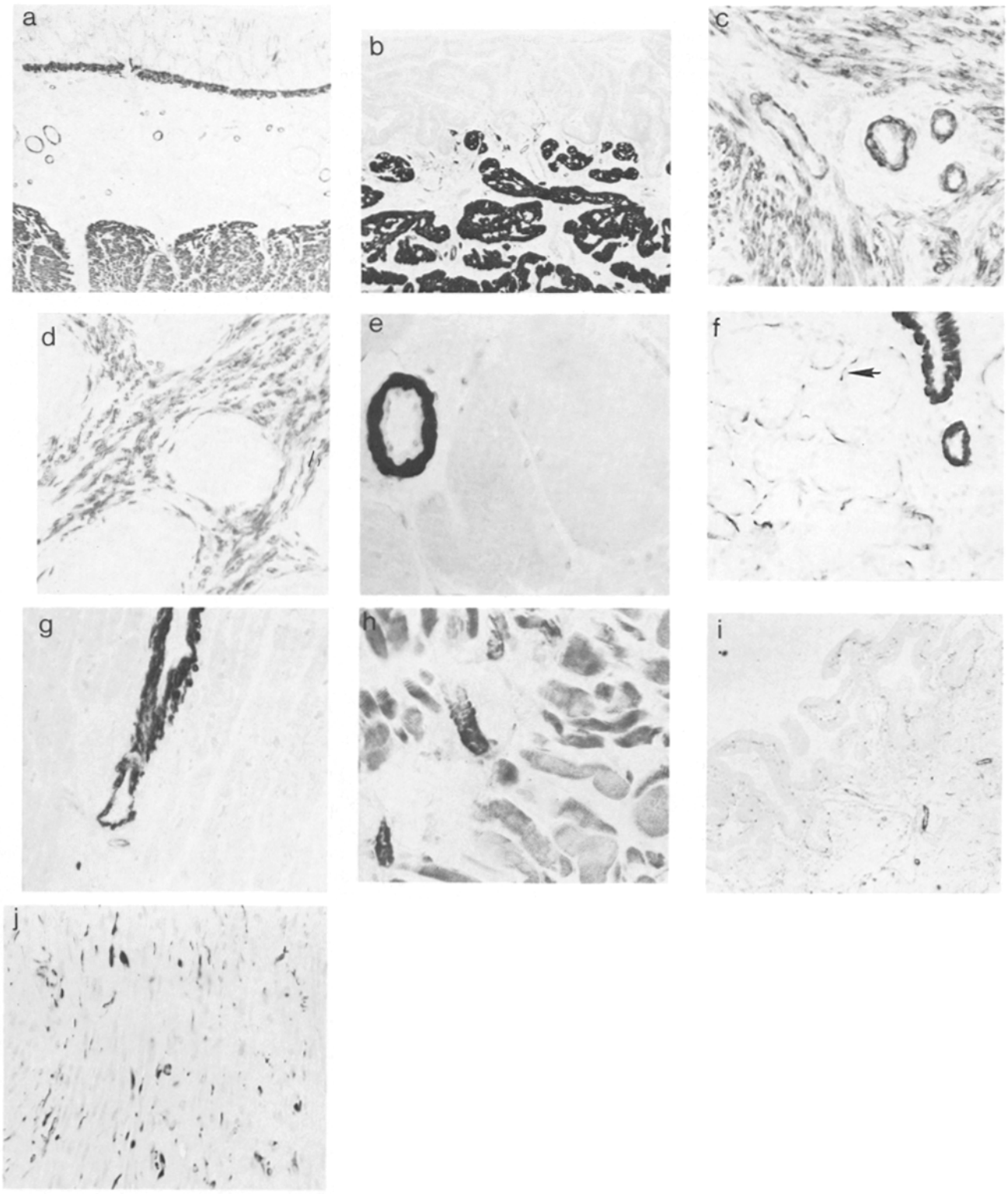

FIGURE 1 Biotin-avidin immunoperoxidase staining of methanol-Carnoy's fixed, paraffin-embedded tissue. (a) Colon, CGA7, × 100; (*b*) gall bladder, CGA7, × 100; (c) myometrium, CGA7, × 400 (note the negative endothelial cells); (*d*) prostate, CGA7, × 400; (e) skeletal muscle, CGA7, × 400 (note positive blood vessel and negative endothelial cells); (*f*) salivary gland, CGA7, × 200 (note the fine staining around glandular acini [arrow] and strong staining of blood vessels); (g) myocardium, CGA7, × 200; (*h*) skeletal muscle, polyclonal rabbit antiactin, × 400; (*i*) gall bladder, monoclonal antivimentin, × 100; (*j*) leiomyoma, monoclonal antivimentin, × 400. CGA7 is used as ascites fluids diluted 1:500 in PBS. The monoclonal antivimentin is used as an ascites fluid diluted 1:1,000 in PBS.

ability to react with CGA7 (data not shown). Increasing the concentration of CGA7 by 10-fold resulted in weak, diffuse cytoplasmic staining of human fibroblasts (Fig. 2 *d*), but this staining was equivalent to that observed with a similar dilution of an antikeratin antibody (Fig. 2e) that does not recognize mesenchymal cells (17, 27). Therefore, increasing the anti-

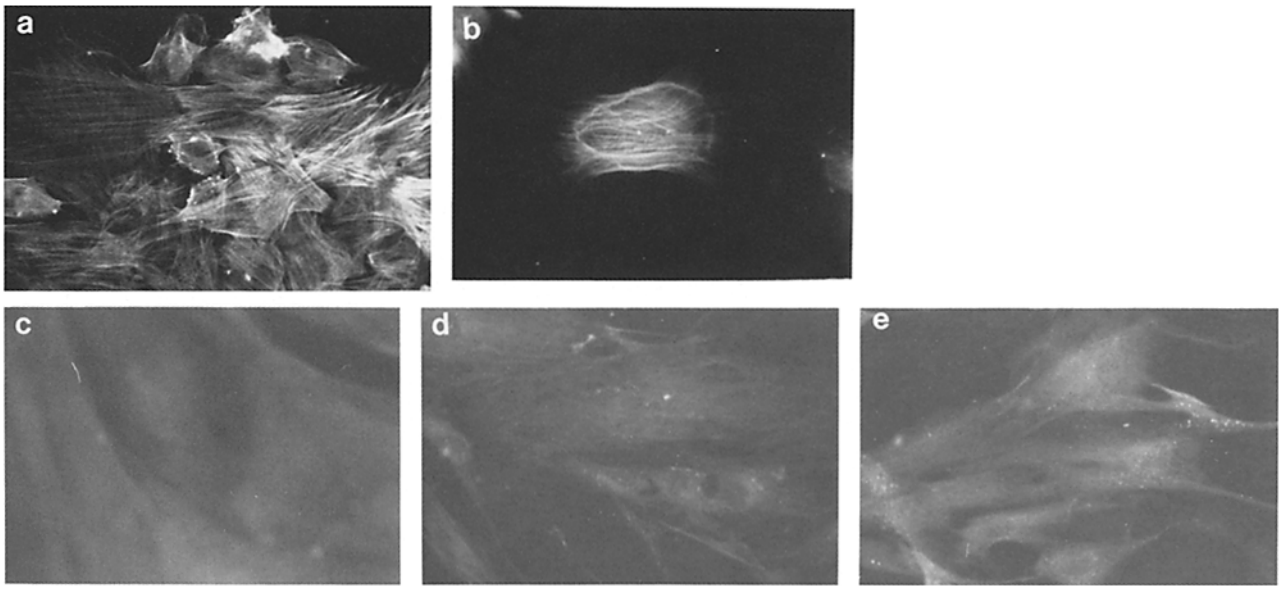

FIGURE 2 Immunofluorescence on cultured cells with CGA7. (*a*) Primary cultures of rat aortic medial cells. × 200, 1:100 dilution of ascites fluid. (*b*) Secondary cultures of human well-differentiated leiomyosarcoma. × 400, 1:100 dilution of ascites fluid. (*c*) Human fibroblasts. × 400, 1:100 dilution of ascites fluid. (*d*) Human fibroblasts. × 200, 1:10 dilution of ascites fluid. (*e*) Human fibroblasts. × 200, monoclonal antikeratin 34βE12 (17, 27), 1:10 dilution of ascites fluid.

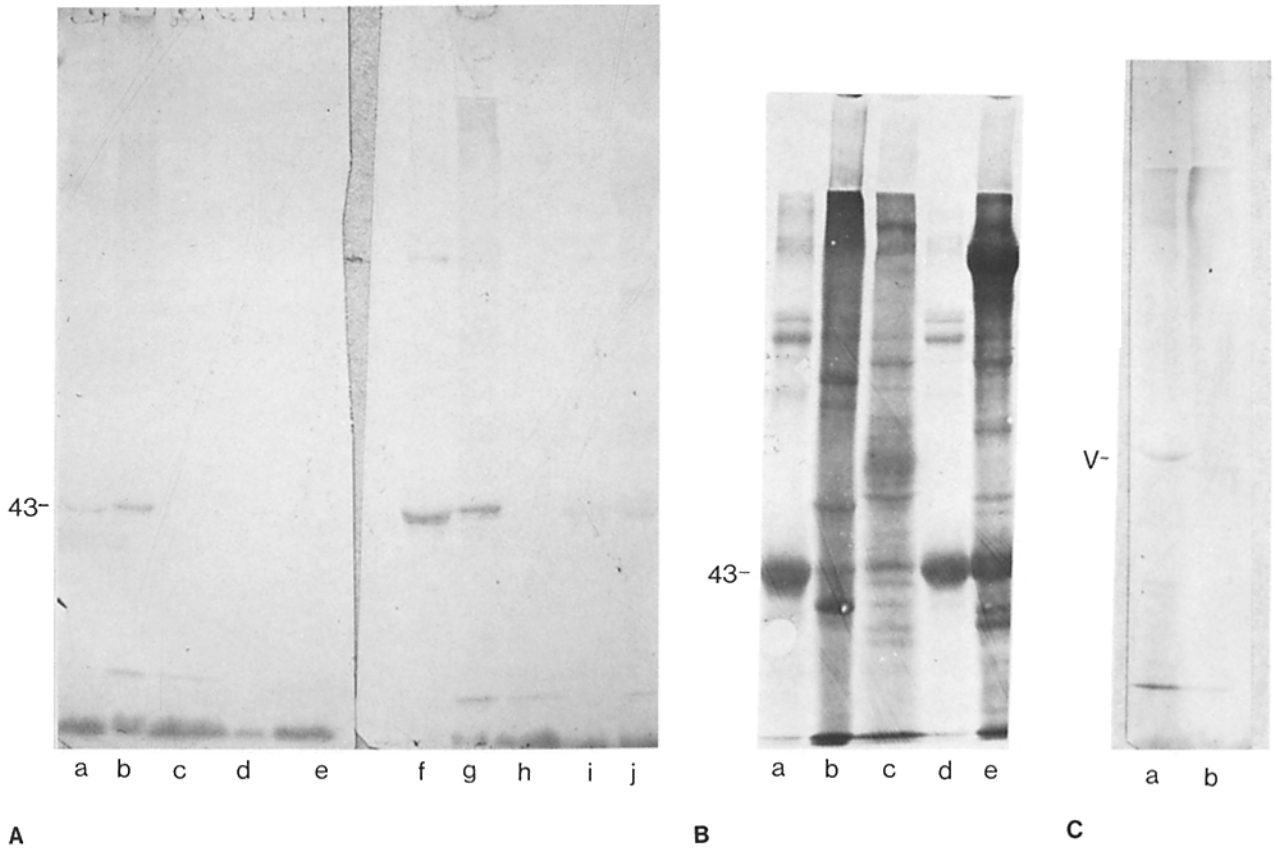

FIGURE 3 Western blots with CGA7 and antivimentin. (*A*) CGA7. (Lanes *a* and *f*) Purified chicken gizzard actin, 20 μg; (lanes *b* and *g*) monkey ileum muscularis solubilized in SDS, ~10 μg; (lanes c and *h*) cultured human fibroblasts solubilized in SDS, ~10 μg; (lanes *d* and *i*) purified rabbit skeletal muscle actin, 20 μg; (lanes *e* and *j*) human skeletal muscle solubilized in SDS, ~20 μg. Lanes a–e, CGA7 ascites diluted 1:100; lanes f–j, CGA7 ascites diluted 1:10. (*B*) Amido black–stained nitrocellulose paper. (Lane *a*) Purified chicken gizzard actin; (lane *b*) monkey ileum; (lane c) human fibroblasts; (lane *d*) purified rabbit skeletal muscle actin; (lane e) human skeletal muscle. The same amount of protein was loaded on this gel as described in *A*. (*C*) Antivimentin ascites fluid diluted 1:100. (Lane *a*) Human fibroblasts; (lane *b*) monkey ileum.

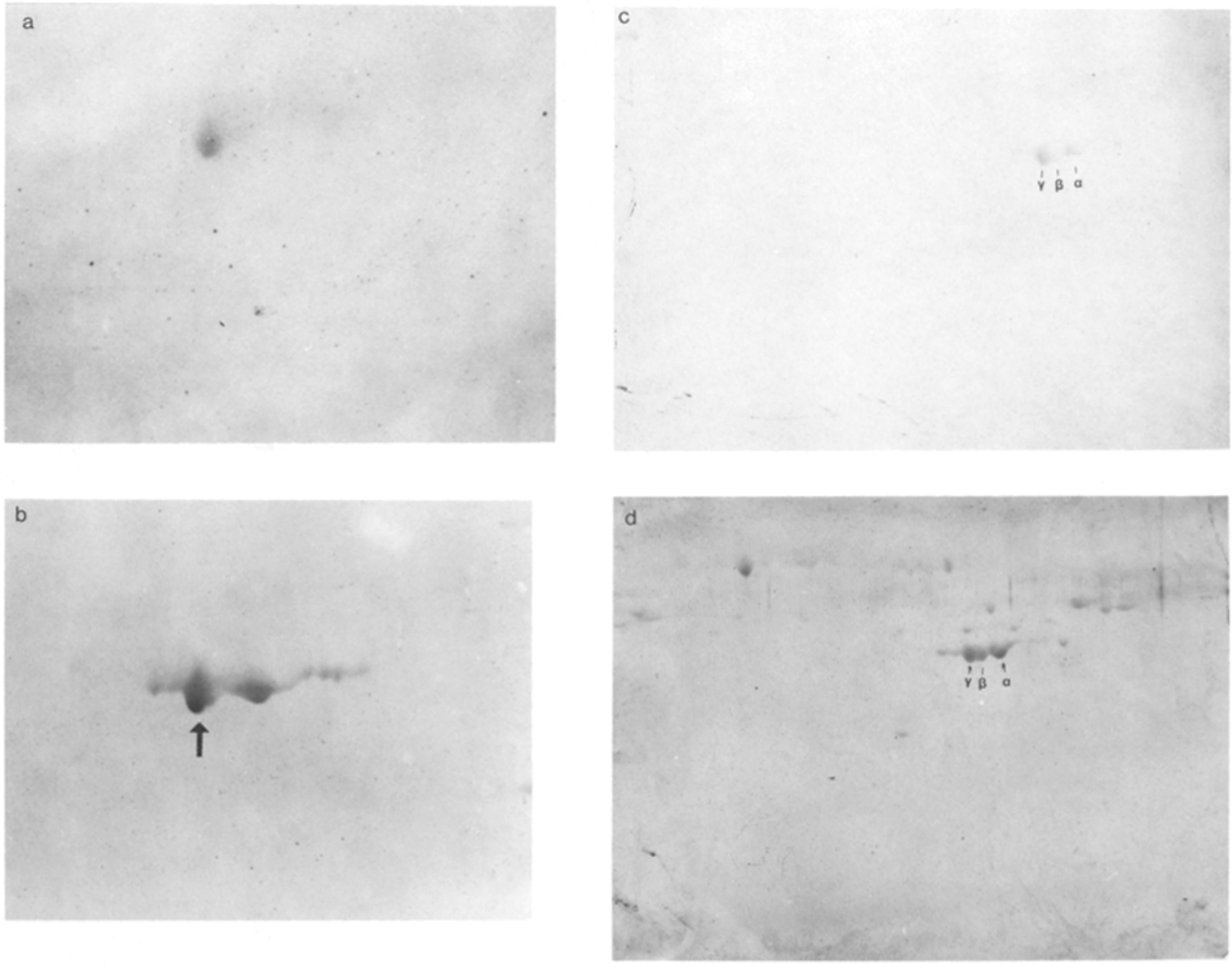

FIGURE 4 Two-dimensional Western blots with CGA7. (*a*) 20 μg of chicken gizzard actin was separated on two-dimensional gels, transferred to nitrocellulose, and incubated with CGA7 ascites fluid diluted 1:100, followed by peroxidase-conjugated anti-mouse IgG. A single spot was detected. (*b*) The same piece of nitrocellulose was then incubated with the rabbit polyclonal antiactin serum diluted 1:200 to detect all actin species. The spot recognized by CGA7 (arrow) is the most basic major actin species in the gel (γ mobility). (c) Rat colon extract (~10 μg) was separated on two-dimensional gels, transferred to nitrocellulose, and incubated with CGA7 ascites fluid diluted 1:100. The antibody recognized α and γ actin. (*d*) Amido black–stained nitrocellulose showing all the proteins in the rat colon preparation. The α-, β-, and γ-actin species are identified on each sample.

body concentration did not result in the recognition of non-smooth muscle cells.

Antigen Identification

The immunofluorescence data suggested that CGA7 recognizes a microfilament-associated protein. We therefore performed immunoblot experiments to see if the antibody recognizes actin. In Western blot experiments, CGA7 detected a 43,000-mol-wt protein in preparations of purified chicken gizzard actin and extracts of monkey ileum but did not recognize purified rabbit skeletal muscle actin or SDS extracts of human skeletal muscle or cultured human fibroblasts (Fig. 3*A*). The lack of recognition of skeletal muscle actin cannot be the result of differences in the amount of actin present on the gels since the Amido black–stained nitrocellulose paper demonstrated similar quantities of chicken gizzard and skeletal muscle actin (Fig. 3, *A* and *B* [lanes *a*, *d*, and *e*]). Additionally, increasing the concentration of CGA7 10-fold resulted in barely detectable recognition of skeletal muscle actin and other skeletal muscle proteins (Fig. 3*A*, lanes *i* and *j*). A negative control using an antivimentin antibody demonstrated that a non–smooth muscle–specific antibody does not recognize actin from human fibroblasts or monkey ileum (Fig. 3*C*). We have always observed that CGA7 recognizes almost the entire width of the actin band from monkey ileum (Fig. 3*A* [lanes *b* and *g*]), but recognizes only a narrow portion of the actin band from chicken gizzard (Fig. 3*A* [lanes *a* and *f*]). The recognition of only a fraction of the chicken gizzard actin raises the possibility that the antibody recognizes a minor contaminant that co-migrates with actin. However, this explanation is unlikely because immunoblots using two-dimensional gels demonstrated that CGA7 recognizes smooth muscle actin isotypes (see below).

These data suggest that CGA7 recognizes smooth muscle specific actin and fails to react with non–smooth muscle actin. To test further this hypothesis, we performed Western blots with purified chicken gizzard actin and extracts of rat colon

muscularis separated on two-dimensional gels. Chicken gizzard predominantly contains a smooth muscle γ isotype, whereas colon muscularis contains both α- and γ-smooth muscle isotypes (9, 29). CGA7 recognizes only γ-actin in chicken gizzard (Fig. 4*a*) but recognizes both α- and γ-actin in extracts of rat colon (Fig. 4*c*). Additionally, in rat aortic media, CGA7 recognizes only α-actin, (Gordon, D., A. M. Gown, A. M. Vogel, and S. M. Schwartz, manuscript in preparation), the predominant actin isozyme in vascular smooth muscle (8). We conclude that CGA7 recognizes smooth muscle-specific actin isotypes.

DISCUSSION

We described here a smooth muscle-specific monoclonal antibody that specifically recognizes smooth muscle actin isozymes. It decorated stress fibers in cultures of early passage smooth muscle cells (Fig. 2) and recognized smooth muscle actin isotypes in immunoblot experiments (Figs. 3 and 4). The recognition of different actin isoforms in different smooth muscle tissues was consistent with the finding that α- and γ-type actins, distinct from skeletal muscle α- and nonmuscle γ-actins, are present in smooth muscle (8, 9, 29).

The biochemical basis for the specificity of CGA7 is unclear. The amino acid sequences of chicken gizzard γ-actin and skeletal muscle α-actin differ at residues 1, 3, 17, 89, 298, and 357 (9). Therefore, any of these variations may determine the epitope recognized by CGA7. We have tried to isolate a proteolytic fragment of chicken gizzard actin recognized by CGA7 but have not yet been successful (Vogel, A. M., unpublished data).

Other investigators have generated antibodies specific for a particular actin isozyme by selective absorption against a particular isozyme (14–16) or by making an antibody to the amino terminal sequence of a particular isozyme (13). These methods have yielded antibodies that specifically recognize skeletal muscle α-actin, γ-actin (both smooth and nonmuscle type), and nonmuscle actin. To our knowledge, this is the first report of a monoclonal antibody that specifically recognizes actin isozymes found in smooth muscle cells.

Clinical and Experimental Uses of CGA7

These antibodies can be used as smooth muscle markers in diagnostic surgical pathology and in experimental studies in atherosclerosis. Anti-intermediate filament protein antibodies are useful markers for specific classes of neoplasms (27, 30–32). The work presented here demonstrates that an antiactin antibody can also function as a tissue-specific reagent. Aside from staining non-neoplastic smooth muscle, CGA7 recognizes leiomyomas and well differentiated leiomyosarcomas (data not shown). It does not recognize carcinomas, melanomas, lymphomas, or non-smooth muscle sarcomas. Small round cell neoplasms, tumors that display no differentiated features, also fail to react with this antibody. We have yet to find a non-smooth muscle tumor recognized by CGA7.

Atherosclerosis is a process characterized by migration and proliferation of smooth muscle cells and infiltration of macrophages and other cells into the intima of blood vessels (33, 34). A smooth muscle cell marker such as CGA7 may allow the exact quantitation of different cell types within atherosclerotic lesions. Preliminary studies indicate that many but not all cells in plaques react with CGA7 (Gown, A., R. Ross, and T. Tsukada, manuscript in preparation). One possible difficulty with such a study is that the antigen recognized by CGA7 may be expressed only in nonproliferating or slowly growing smooth muscle cells and that growth may result in the loss of the smooth muscle specific marker. The finding that smooth muscle cells cultured in vitro lose reactivity with CGA7 supports this hypothesis. More work is required to evaluate the usefulness of these antibodies in the study of atherosclerosis.

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