

Immunolocalization of the Gamma Isoform of Nonmuscle Actin in Cultured Cells

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Abstract. In many vertebrate nonmuscle cells, the microfilament subunit protein, actin, exists as two isoforms, called β and γ , whose sequences differ only in their amino-terminal regions. We have prepared a peptide antibody specifically reactive with the amino-terminal sequence of γ actin. This antibody reacted with nonmuscle actin as determined by Western blots of SDS gels, and reacted with the γ , but not the β , nonmuscle actin isoform as shown by Western blots of isoelectric focusing gels. In immunofluorescence experiments, the γ peptide antibody stained microfilament bundles, ruffled edges, and the contractile ring

of a variety of cultured cells, including mouse L cells, which have previously been reported to contain only the β actin isoform (Sakiyama, S., S. Fujimura, and H. Sakiyama, 1981, *J. Biol. Chem.*, 256:31–33). Double immunofluorescence experiments using the γ peptide antibody and an antibody reactive with all actin isoforms revealed no differences in isoform localization. Thus, at the level of resolution of light microscopy, we have detected the γ actin isoform in all microfilament-containing structures in cultured cells, and have observed no subcellular sorting of the nonmuscle actin isoforms.

ACTIN, a major constituent of eukaryotic cells, is a cytoskeletal protein whose amino acid sequence has been extraordinarily conserved throughout evolution. To date, six different actin isoforms have been described in vertebrates, including two forms, β and γ , which are present in most nonmuscle cells (41). Each of the six isoforms is the product of a separate gene (7). Amino acid sequence information has shown that all of these isoforms display >90% sequence homology; most of the amino acid substitutions are present in the amino terminus, which has a unique sequence in each isoform (41).

Expression of actin isoforms is known to be highly variable. Nonmuscle cells and tissues generally contain β and γ actins, in ratios ranging from 6:1 to 1:8 (β : γ) in different tissues and cell types (9, 42). Two cell types have been described as containing little or no γ actin: mouse L cells (33) and human leukemic T cells (20). Recent evidence indicates that certain physiological changes in cultured cells can be correlated with alterations in a specific actin isoform. For example, altered forms of β actin have been found in mutant KB cells which have acquired resistance to cytochalasin B (40) and in chemically transformed human fibroblasts (17). In addition, isoforms other than nonmuscle β and γ have been detected in some nonmuscle cells; a more acidic (δ) actin in transformed sarcoma 180 cells (3), and the smooth muscle α actin isoform in several cell lines of fibroblast origin (21, 38, 43). The expression of smooth muscle α actin has previously been found to be modulated according to the transformation or differentiation state of fibroblasts (8, 21, 38, 43).

Although six actin isoforms exist and are regulated in their tissue-specific expression, the biological significance of actin isoforms remains unknown. It is possible that these highly similar proteins are differentially localized *in vivo*. The work of McKenna et al. (25) demonstrates that microinjection of actin into cultured cells does not result in a differential localization of the actin isoforms introduced; however, nothing is known regarding the distribution of the endogenous isoforms in these or other cells in culture. Accordingly, we examined the distribution of actin isoforms in cultured cells and chose, for the study described in this paper, the simplest case; that is, the localization of γ actin in cultured cells which contain both the β and γ isoforms of actin.

Materials and Methods

Peptide Synthesis

Peptides of sequence Acetyl-(Glu)₃-Ile-(Ala)₂-Leu-Val-Ile-Asp-Asn-Gly-Ser-Gly-Tyr-COOH for the γ peptide and Acetyl-(Asp)₃-Ile-(Ala)₂-Leu-(Val)₂-Asp-Asn-Gly-Ser-Gly-Tyr-COOH for the β peptide, corresponding to the NH₂ terminus of vertebrate nonmuscle γ and β actins (except with an added Tyr at each COOH terminus) were synthesized by the Merrifield solid-phase method as modified by Stewart (37). Tertiary butyloxycarbonyl (t-BOC)¹ amino acids were purchased from Vega Biotechnologies, Inc. (Tucson, AZ). Sequanal grade trifluoroacetic acid was purchased from Pierce Chemical Co. (Rockford, IL). All other analytical grade reagents used were obtained from Mallinckrodt Inc. (Paris, KY). ¹⁴C-Glycine purchased from Amersham Corp. (Arlington Heights, IL) was t-BOC derivatized by the protocol of Stewart (37) and incorporated

1. *Abbreviations used in this paper:* ELISA, enzyme-linked immunosorbent assay; IEF, isoelectric focusing; KLH, Keyhole limpet hemocyanin; t-BOC, tertiary butyloxycarbonyl.

into the fourth residue added, in order to provide a radioactive tracer for the peptides (final specific activity was 50 cpm/nmol). Peptides were purified on a P-4 gel filtration column (Bio-Rad Laboratories, San Francisco, CA). Coupling of γ actin peptide to Keyhole limpet hemocyanin (KLH) was through the tyrosine residue at the COOH terminus of the peptide. Immunogen consisted of γ peptide-KLH conjugate, which was prepared using 2 mg of peptide in 0.16 M sodium borate, pH 9, 0.15 M sodium chloride, 0.5 mg of KLH, and 0.16 mg *bis*-diazobenzidine as the coupling reagent (2). Coupling efficiencies of 80–95% were ascertained by scintillation counting.

Preparation of Antipeptide Antibodies

Two female, New Zealand White rabbits were immunized subcutaneously according to the following schedule: 200 μ g of peptide-KLH conjugate in Freund's complete adjuvant on day 0, 100 μ g of the same conjugate in Freund's incomplete adjuvant on day 14, and 100 μ g booster injections in Freund's incomplete adjuvant every 5 to 6 wk thereafter. Animals were bled at 7–10-d intervals after the second injection. Ammonium sulfate-fractionated sera were used in some experiments; rabbit antisera were precipitated with 40% ammonium sulfate, resuspended in one-half of the original serum volume with phosphate-buffered saline (PBS), and dialyzed extensively against this same buffer before use. Although all bleeds from both rabbits showed the same specificity, only those bleeds which had been fully characterized by immunoblot analysis were used for immunofluorescence experiments.

The mouse monoclonal antibody, C4, was prepared using chicken gizzard smooth muscle actin as the immunogen (22). C4 was previously shown to be reactive with all actin isoforms. A preparation of C4 ascites fluid (1 mg/ml) was diluted as indicated for use in each experiment.

Preparation of Cell and Tissue Extracts and Purified Actins

To prepare extracts of cultured cells, confluent cultures were scraped from the plates with a rubber policeman, centrifuged at 1,200 rpm for 5 min, washed once with PBS, and centrifuged again. The pellet was resuspended in SDS gel sample buffer (0.5 ml/10 μ l of pellet) or isoelectric focusing (IEF) sample dilution buffer (9.5% urea, 5% β -mercaptoethanol, 1.6% Triton X-100; 150 μ l/10 μ l of pellet) and stored at -70°C until electrophoresis.

Heart and brain extracts were prepared by rinsing organs from adult female rats in ice-cold PBS, homogenizing in a ground glass homogenizer, and centrifuging at 3,000 g for 5 min at 4°C . The protein content of the supernatants was measured (23) before samples were prepared for electrophoresis.

Bovine spleen actin was prepared by the procedure of Carlsson et al. (6). Porcine brain actin was obtained using the method of Nagata et al. (27), except that whole brains were used as starting material. Chicken muscle actin was prepared from acetone powders as described by Spudich and Watt (36).

Immunological Assays

The enzyme-linked immunosorbent assay (ELISA) was performed as described by Atherton and Hynes (1). Coating antigens used were either purified porcine brain actin (3 μ g/well), purified bovine spleen actin (2 μ g/well), or β or γ peptide coupled to bovine serum albumin (BSA) (1 μ g/well). Conjugation of peptide to BSA was performed essentially as for KLH, except that 10 mg of β or γ actin peptide was used per 0.5 mg BSA. The competitive ELISA procedure used was that of Raugi et al. (31).

Western blots of SDS polyacrylamide gels were performed according to Bulinski et al. (5). The procedure used for the Western blots of IEF tube gels will be described in detail elsewhere (Otey, C. A., and J. C. Bulinski, manuscript submitted for publication). Briefly, samples and gels were prepared as described by O'Farrell (29), but with a mixture of ampholines at concentrations of 4% (vol/vol) of pH 5–7, and 1% of pH 3.5–10 (LKB Instruments, Inc., Gaithersburg, MD). The tube gels were treated to remove the urea and ampholytes and were then blotted and immunostained normally. Some gels were stained with Coomassie Brilliant Blue before blotting and immunostaining. This protocol

was useful because it enabled us to make a direct comparison between the immunostained bands, which appeared black on the nitrocellulose sheet, with the protein-stained bands, which appeared blue.

Cell Culture and Immunofluorescent Staining

All cells were grown at 37°C in a humid atmosphere containing 5% CO_2 , in media obtained from GIBCO (Grand Island, NY) supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT). TC-7 (African green monkey kidney), Chinese hamster ovary, and PtK-1 (rat kangaroo kidney) cells were cultured in Dulbecco's modified Eagle's medium. Mouse L (mouse fibroblast) and LK-174 cells (a derivative of L cells transfected with, but not expressing, the cardiac α actin gene [14], which were obtained from Dr. Robert Hickey, Albert Einstein College of Medicine) were grown in F-10 medium and HAT-F-10 medium, respectively. All cells were grown in plastic tissue culture dishes or were seeded onto glass coverslips for immunofluorescence.

For the cell-spreading experiments, confluent L cell cultures were treated briefly with trypsin solution (0.25% trypsin [Sigma Chemical Co., St. Louis, MO], 0.01% EDTA in Earle's balanced salt solution [GIBCO]), and seeded onto sterile 22-mm glass coverslips. The coverslips were fixed in -20°C methanol for 5 min at intervals from 20 min to 6 h after replating, and stored in PBS until stained.

Fixation and immunofluorescent staining were performed as described by Bulinski and Boris (4) except using ammonium sulfate-fractionated antisera at dilutions of 1:40 to 1:200. In some experiments, an alternate method of immunostaining was used. Cells fixed on coverslips were placed in 100-mm petri dishes containing 10 ml of γ peptide antibody diluted 1:12,000 in PBS containing 1% BSA. The dishes were placed on a rocker at low speed for 1 h, then rinsed and treated with secondary antibody as usual. C4, the mouse monoclonal antibody reactive with all actin isoforms, was used at dilutions of 1:60 to 1:80 in PBS. Rhodamine- and fluorescein-conjugated secondary antibodies were used at dilutions of 1:40. Immunostained cells were observed with a Nikon Optiphot epifluorescence microscope, using a Zeiss 40 \times or 63 \times planapochromat objective, and were photographed on Kodak Tri-X film. Exposure times were 3–10 s for single-stained and 5–20 s for double-stained cells. To prevent bleed-through, double-stained cells were photographed on the rhodamine channel (filter cube G), bleached until no red light was observed (~ 5 min), and then photographed on the fluorescein channel (filter cube B₂).

Results

Preparation and Characterization of Peptide Antibody

The two nonmuscle actin isoforms, β and γ , co-exist in most nonmuscle cells and tissues. An investigation of the cellular localization of γ actin would be possible with antibodies that react specifically with the γ , but not the β , isoform. As shown in Table I, γ and β actins have four amino acid sequence differences at their amino termini; otherwise their sequences are identical (41). Therefore, we prepared a synthetic peptide containing 15 amino acids; the NH_2 -terminal sequence of this peptide corresponded to the NH_2 -terminal sequence of γ actin (see Materials and Methods for details). The γ actin peptide was conjugated to KLH and used as an immunogen in rabbits. Antibodies obtained were tested for their reactivity in an ELISA. As shown in Fig. 1, curve *a*, the γ peptide antibody reacted strongly with spleen actin, which contains mostly γ and β actins. The antibody reactivity was greater than background levels even at a 1:25,000 dilution of antibody. In control experiments antibody reactivity was not affected by pre-absorption with a molar excess of β peptide (curve *b*), but

Table I. Amino Terminal Sequence of Nonmuscle Actins

	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Beta	Ac-Asp	Asp	Asp	Ile	Ala	Ala	Leu	Val	Val	Asp	Asn	Gly	Ser	Gly
Gamma	Ac-Glu	<u>Glu</u>	<u>Glu</u>	<u>Glu</u>	Ile	Ala	Ala	Leu	Val	<u>Ile</u>	Asp	Asn	Gly	Ser

Residues in which β and γ isoforms differ are underlined. Residue 2 is acetylated (Ac-) in each actin. Sequence information and numbering convention (to correspond to the muscle actins which are longer by one residue) are from Vandekerckhove and Weber (41). No sequence differences between β and γ actins outside of the NH_2 terminus have been detected (41); the sequence of β actin has been verified by nucleotide sequencing of genomic clones (18).

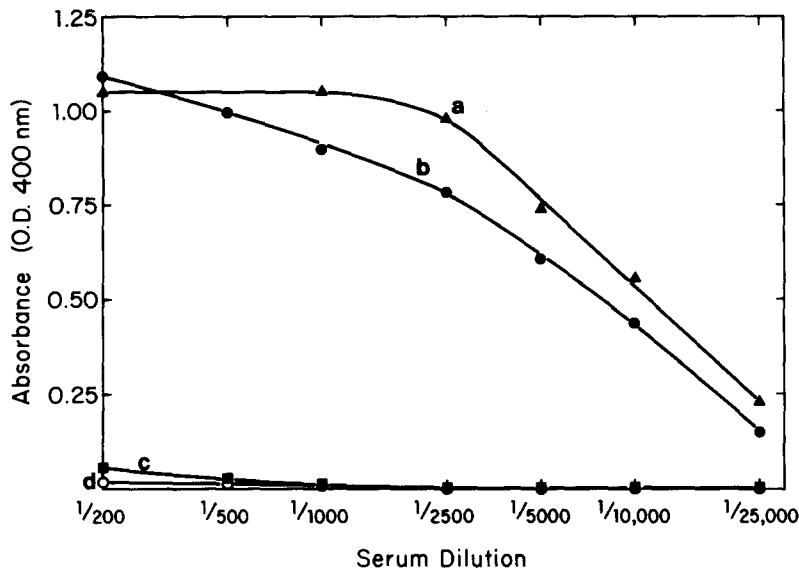


Figure 1. ELISA of γ peptide antibody against non-muscle actin. Ammonium-sulfate fractionated antisera were diluted as shown and incubated in polystyrene microtiter plates coated with purified bovine spleen actin ($2 \mu\text{g}/\text{well}$). An alkaline phosphatase-conjugated goat anti-rabbit second antibody followed by reaction with the phosphatase substrate, *p*-nitrophenyl phosphate, was used to detect the binding of actin antibodies. Curve *a* (solid triangles), γ peptide antibody; curve *b* (solid circles), γ peptide antibody preincubated with β actin peptide (10^{-5} M for 60 min at 37°C); curve *c* (solid squares), γ peptide antibody preincubated with γ actin peptide (10^{-5} M for 60 min at 37°C); curve *d* (open circles), preimmune serum from rabbit subsequently injected with γ actin peptide. Curves *a*–*c* represent the reactivity of antibody from an average bleed from one of the rabbits immunized with γ peptide.

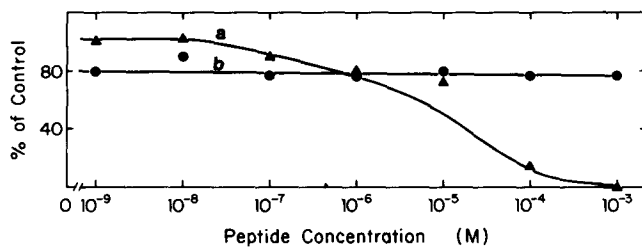


Figure 2. Competitive ELISA of γ peptide antibody. Conditions were identical to those used for the ELISA in Fig. 1, except that the antibody was preincubated with the indicated concentration of β or γ peptide. The antibody was used at a dilution of 1:5,000 which gave an A_{400} of 0.75–1.00 in control samples which were not incubated with peptide. Curve *a* (solid triangles), γ peptide antibody preincubated with γ peptide; curve *b* (solid circles), γ peptide antibody preincubated with β peptide.

preimmune sera (curve *d*) and antibody that was pre-absorbed with a molar excess of γ peptide (curve *c*) or with purified nonmuscle actin (not shown) did not react in the ELISA.

The specificity of the antibody for the γ actin NH_2 -terminal peptide was tested by a competitive ELISA, in which the γ peptide antibody was pre-incubated with either the γ or the β peptide and then added to the actin-coated microtiter wells. As shown in Fig. 2, curve *a*, the γ peptide completely inhibited the reactivity of the γ peptide antibody. However, the β peptide, even at 100-fold greater concentration, did not affect the reactivity of the antibody (curve *b*). Thus, the γ peptide antibody showed great specificity for the peptide to which it was elicited.

Western blot analysis was used to demonstrate the specificity of the antibody for actin. As shown in Fig. 3, *c* and *d*, the antibody only reacted with material of the same gel mobility as actin in samples of purified spleen actin and spleen extract. Antibody from rabbits subsequently injected with γ peptide (Fig. 3, lanes *e* and *f*) and antibody pre-absorbed with γ peptide (lanes *g* and *h*) showed no reactivity. Thus, the γ peptide antibody showed strong reactivity with nonmuscle actin, and displayed specificity for the NH_2 -terminal sequence of γ actin.

To determine the actin isoform specificity of the γ peptide

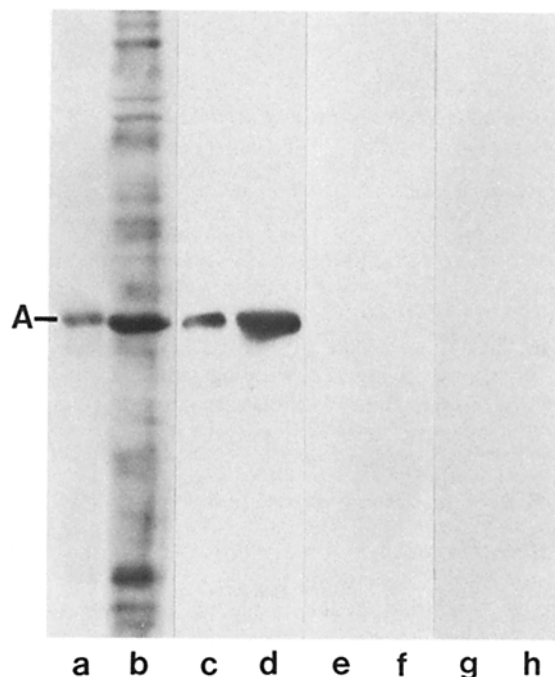


Figure 3. Immunoblot of SDS gels stained with γ peptide antibody. Quadruplicate samples were electrophoresed on 10% polyacrylamide gels and transferred to nitrocellulose. Lanes *a*, *c*, *e*, and *g* contain $5 \mu\text{g}$ of purified bovine spleen actin. Lanes *b*, *d*, *f*, and *h* contain $20 \mu\text{g}$ of bovine spleen extract. Lanes *a* and *b* were stained for protein with Coomassie Brilliant Blue; lanes *c* and *d* were stained with a 1:5,000 dilution of γ peptide antibody; lanes *e* and *f* were stained with a 1:5,000 dilution of preimmune serum; lanes *g* and *h* were stained with a 1:5,000 dilution of γ peptide antibody preabsorbed with 10^{-4} M γ actin peptide. Antibody binding was visualized by incubating blots in peroxidase-labeled goat anti-rabbit IgG followed by the substrate, 4-chloro-1-naphthol. *A*, Gel mobility of actin.

antibody, extracts of rat brain (which contains both nonmuscle β and γ isoforms) and rat heart (which contains cardiac α , and nonmuscle β and γ actins) were resolved by IEF and subjected to immunoblotting. Because the isoelectric points of β and γ actins differ by only 0.02 pH units (9), we wished to avoid any loss of resolution which might occur in running

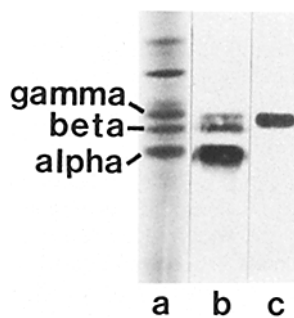


Figure 4. Immunoblot of IEF gels stained with γ peptide antibody. Combined extracts of rat brain and rat heart were focused and transferred to nitrocellulose (see Materials and Methods). Lane *a* shows a gel stained with Coomassie Brilliant Blue. Lane *b* was stained with C4 actin monoclonal antibody diluted 1:1,000. Lane *c* was stained with γ peptide antibody diluted 1:12,000. The positions of the α , β , and γ actins are labeled.

the IEF gels in the second (SDS) dimension. Therefore, we elected to blot the IEF gels directly and stain the blots with antibody. As shown in Fig. 4*c*, the antibody showed specificity for the γ actin band of the organ extract. As with other antibodies, more stained bands could be observed in Western blot experiments by progressively decreasing the dilutions of antibody used (1:5,000–1:500), though these other bands were never as bright as the γ actin band. Staining at the positions of the β and cardiac α actins was attributed to nonspecific binding, since this staining was only observed at dilutions lower than 1:5,000; at these low dilutions of antibody, other background bands were equally stained, including all of the tubulin bands. It was, of course, possible that the apparently specific staining of the γ actin band reflected the fact that γ actin transferred to nitrocellulose much more efficiently than the other actin isoforms. To determine if all three isoforms had been transferred to the nitrocellulose, parallel blots were stained with C4, a monoclonal antibody reactive with all actin isoforms. As shown in Fig. 4*b*, staining with C4 antibody revealed three distinct bands with isoelectric positions corresponding to those of α , β , and γ actins, thus indicating that all three isoforms were transferred efficiently to the nitrocellulose. The Western blot experiments with IEF gels demonstrate that the γ peptide antibody showed specific reactivity with the γ , and not the β , isoform of nonmuscle actin over a range of usable antibody concentrations.

Because the first three residues at the NH_2 terminus of the γ peptide (Acetyl-Glu-Glu-Glu-) are identical to those in the NH_2 -terminal sequences of the two smooth muscle actins, α (Acetyl-Glu-Glu-Glu-Asp-) and γ (Acetyl-Glu-Glu-Glu-Thr-), we also tested the reactivity of the γ peptide antibody against these isoforms, using Western blots of IEF gels. As expected, we found that the γ peptide antibody reacted strongly with both of the smooth muscle actins (data not shown). As we wished to use our peptide antibody for the immunolocalization of only a single actin isoform (nonmuscle γ), it was important to select for these experiments cells which did not express either of the cross-reactive smooth muscle isoforms. Extracts of four cell lines (Chinese hamster ovary, PtK-1, TC-7, and L) were analyzed by IEF immunoblotting using the generally reactive actin antibody, C4, and a muscle-selective actin antibody (22). In every case, the C4 antibody revealed only two actin species whose isoelectric positions corresponded to those of the β and γ isoforms, and no smooth muscle isoforms were detected with the muscle-selective actin antibody (data not shown). The presence of only the nonmuscle β and γ actin isoforms permitted us to use the γ

peptide antibody to specifically localize the nonmuscle γ isoform in each of the four cell types we examined.

Immunolocalization of γ Actin

The γ peptide antibody was used in indirect immunofluorescence experiments to localize γ actin in a variety of cultured cells. As seen in Fig. 5, the staining patterns obtained were reminiscent of patterns observed with other anti-actin antibodies. Stress fibers in every cell were stained intensely, indicating that all of the cells contained nonmuscle γ actin. As shown in the micrograph of Chinese hamster ovary cells in Fig. 5, *a* and *b*, every fiber visible in the phase-contrast image was brightly stained in the corresponding fluorescent image. No stress fibers were detected by phase-contrast microscopy which were not stained with the γ peptide antibody. Similar patterns of actin staining were seen with both human undifferentiated myoblasts and human fibroblasts (data not shown). In colonies of TC-7 cells, bright staining was often observed along the borders of adjoining cells (data not shown). PtK-1 cells, however, were more brightly stained around the periphery of the colony than between neighboring cells (Fig. 5, *c* and *d*). In colonies of HeLa cells, which do not contain stress fibers, bright edge staining was also seen (data not shown). Thus, the γ peptide antibody labeled actin-containing structures in all cell types examined. The staining patterns observed were the result of antibodies binding to the amino terminus of γ actin, because preimmune antibody (Fig. 5*e*), antibody pre-absorbed with γ peptide (Fig. 5*f*) or pre-absorbed with bovine nonmuscle actin (Fig. 5*g*) all showed only dim, diffuse staining.

To compare the distribution of γ actin to that of total actin (γ + β isoforms) in cultured cells, TC-7 cells were stained by double-indirect immunofluorescence with γ peptide antibody and with the C4 antibody. In many experiments in which hundreds of cells were carefully examined, no differences between the distribution of γ actin and that of total actin could be detected. As shown in Fig. 6, all actin-containing structures which stained with the C4 antibody (Fig. 6, *b* and *e*) also stained with the γ peptide antibody (Fig. 6, *a* and *d*).

Previous work by Nigg et al. (28) demonstrated that when peptide antibodies are used in immunofluorescence or Western blots at high concentrations (10–20 $\mu\text{g}/\text{ml}$ of affinity-purified antibody in their case), cross-reactivity with other proteins containing related sequences may result. To be certain that we were detecting only γ actin with our peptide antibody, we repeated the immunofluorescent staining, using a dilution of the γ peptide antibody identical to that which we have previously shown (Fig. 4*c*) to be specific for the γ isoform in Western blot experiments (i.e., 1:12,000). Immunofluorescent staining with this low concentration of antibody was accomplished by staining in a large volume (10 ml) of antibody solution, just as we have used for Western blots. Fig. 6*f* shows a micrograph of cells stained exactly like the blots shown in Fig. 4*c*; in fact, the same preparation of antibody was used in both cases. In a comparison of Fig. 6, *f* and *g*, no differences in actin localization were observed when the low-concentration antibody was used with the C4 antibody in double-indirect immunofluorescence experiments: both antibodies stained the same stress fibers in interphase cells and also the contractile rings in dividing cells (data not shown). Indeed, under all conditions used, such as alternate fixation

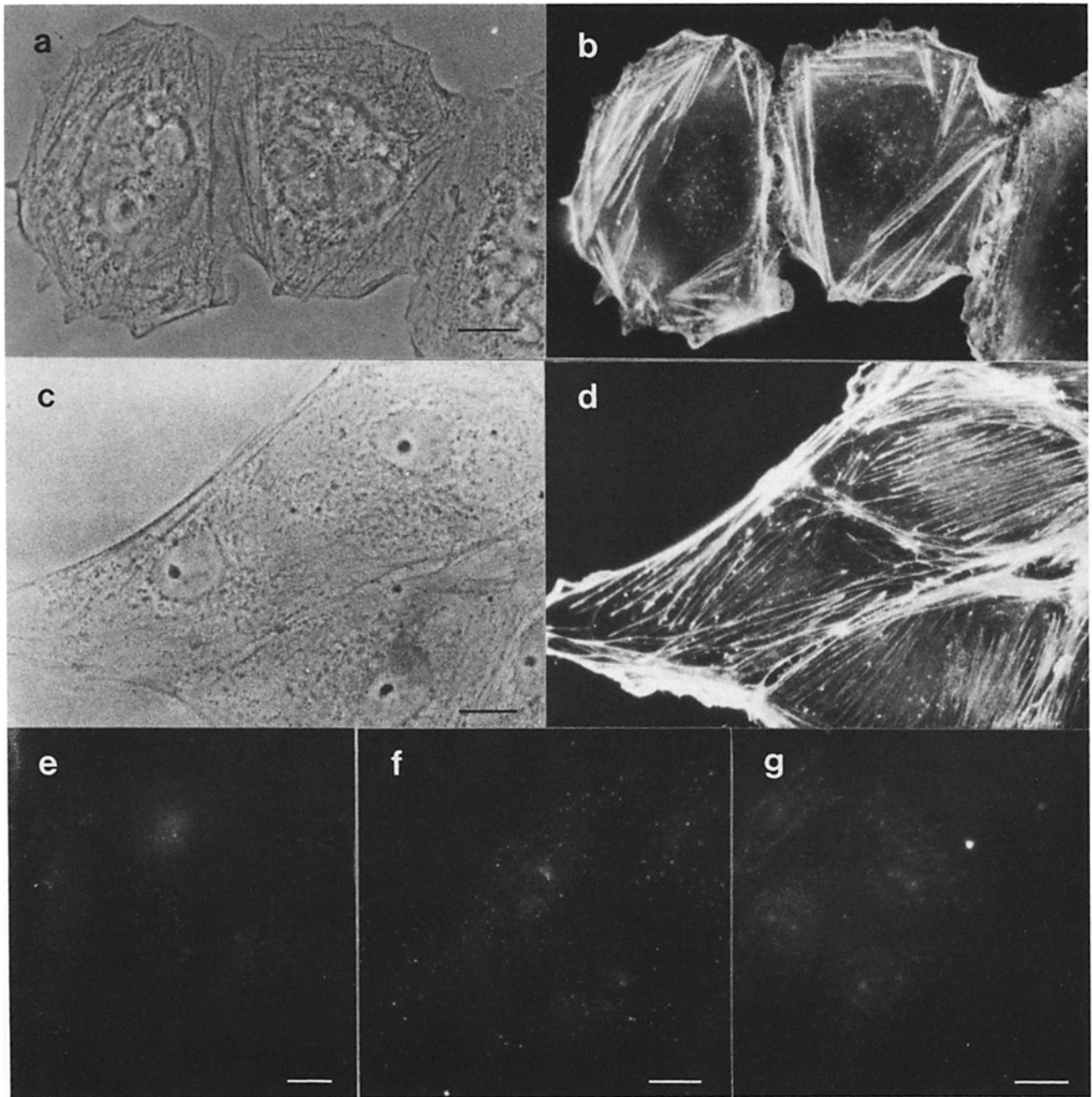


Figure 5. Indirect immunofluorescence localization of nonmuscle γ actin. Staining was performed on methanol-fixed cells with a 1:40 dilution of γ peptide antibody followed by rhodamine-conjugated goat anti-rabbit IgG. (a and b) Phase-contrast and corresponding fluorescence image of Chinese hamster ovary cells; (c and d) phase-contrast and corresponding fluorescence image of interphase rat kangaroo kidney cells (PtK-1); (e) fluorescence image of African green monkey kidney (TC-7) cells stained with preimmune sera; (f and g), fluorescence image of TC-7 cells stained with γ peptide antibody preabsorbed with (f) 10^{-4} M γ peptide or (g) with 10^{-4} M bovine spleen actin for 60 min at 37°C . Bars, $10\ \mu\text{m}$.

(glutaraldehyde or formaldehyde), reversal of the fluorochromes (rhodamine-conjugated secondary antibody used for the γ peptide antibody rather than for C4), or higher or lower concentrations of primary and secondary antibodies, no differences in the distribution of γ and total actin were observed.

Detection of α Actin in Mouse L Cells

All nonmuscle cells examined to date, with a few rare exceptions, have been found to contain both γ and β nonmuscle actin isoforms in varying ratios. One of the exceptions is the

L cell line, derived from mouse fibroblasts, in which only the β isoform has been detected by two-dimensional gel electrophoresis (33). We wished to determine whether or not nonmuscle γ actin could be detected in L cells by the more sensitive immunological techniques of Western blotting and indirect immunofluorescence, using our γ peptide antibody. As shown in Fig. 7, material reactive with γ peptide antibody was observed in Western blots of SDS gels (Fig. 7b). No γ band was observed in protein-stained IEF gels (Fig. 7c). However, in immunoblots of IEF gels, a band reactive with γ

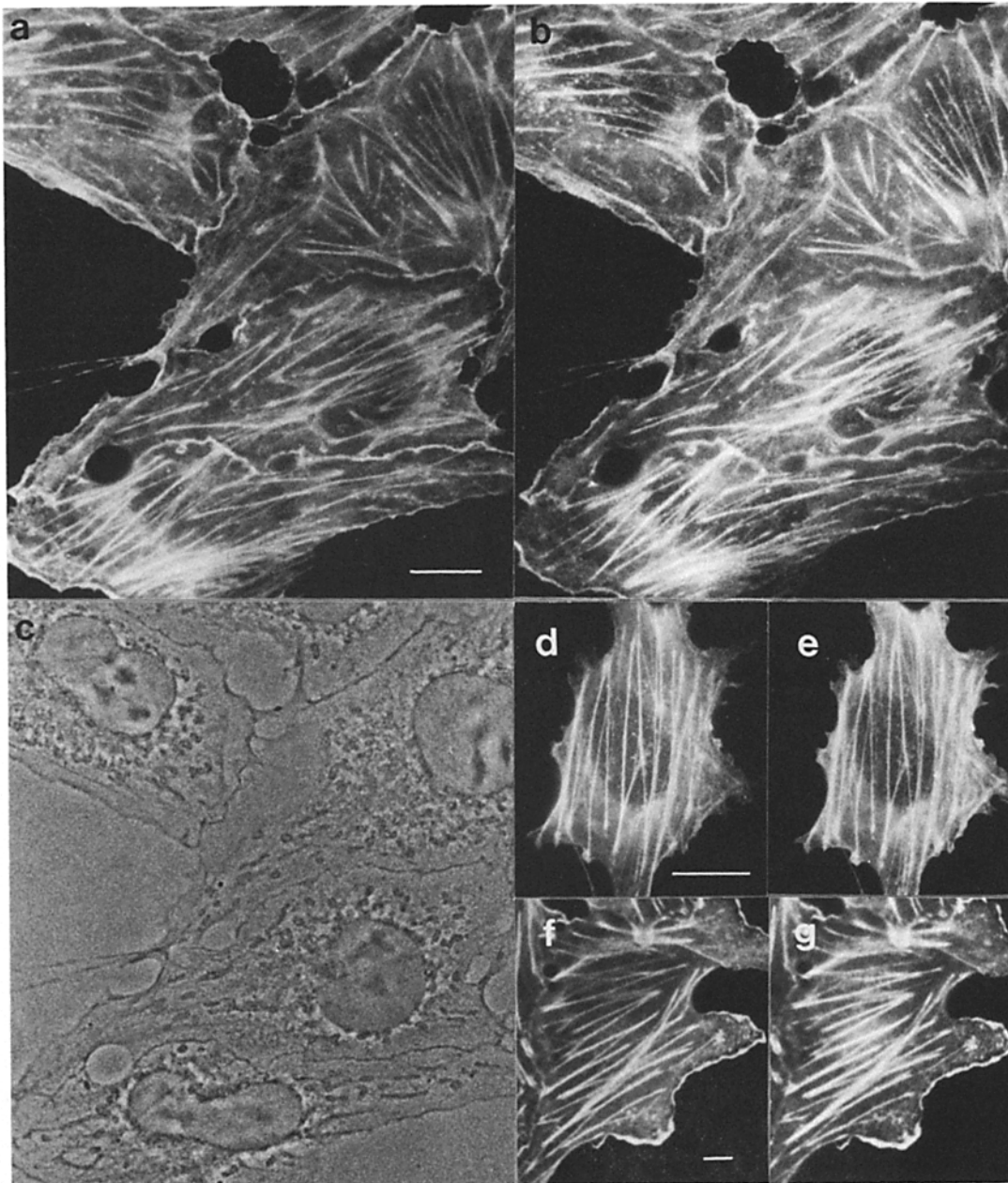


Figure 6. Simultaneous immunolocalization of nonmuscle γ actin and total cellular actin. Indirect immunofluorescent staining of methanol-fixed TC-7 cells with (a and d) γ peptide antibody at a 1:40 dilution or (f) at a 1:12,000 dilution, followed by a fluorescein-conjugated goat anti-rabbit IgG; and (b, e, and g) monoclonal C4 actin antibody (1:60 dilution) followed by a rhodamine-conjugated goat anti-mouse IgG. (c) Phase-contrast image of cells shown in a and b. Bar, 10 μ m.

peptide antibody was clearly visualized (Fig. 7e). Although γ actin is present in very low amounts relative to β actin, L cells do contain this actin isoform.

Since γ actin is a minor isoform in L cells, one might expect it to be segregated into a few minor structures in all cells or to be expressed by only a few cells. We investigated these possibilities by staining L cells with the γ peptide antibody in indirect immunofluorescence experiments. The staining pattern typically observed was that of brush-like structures located above and below the nucleus. In some cells, stress fibers were also observed. All of the cells stained brightly and with roughly equal intensity. This staining pattern can be clearly visualized in the micrograph of a multinucleate giant cell

shown in Fig. 8, a and b. Giant cells are common in L cell cultures and, as they are extremely large and well-flattened, they can be used to obtain immunofluorescence images of unusual detail and clarity. When stained with the γ peptide antibody, giant L cells displayed overlapping arrays of very fine fibers (Fig. 8b) and often contained as many as four or five brush-like structures per cell. However, as multinucleate cells differ from their normal counterparts in many respects, we do not know if this type of γ actin organization is a feature unique to giant L cells or if it is also found, but not resolved clearly, in mononucleate L cells.

To observe any differences between the distributions of γ actin and total actin in vivo, we examined mononucleate L

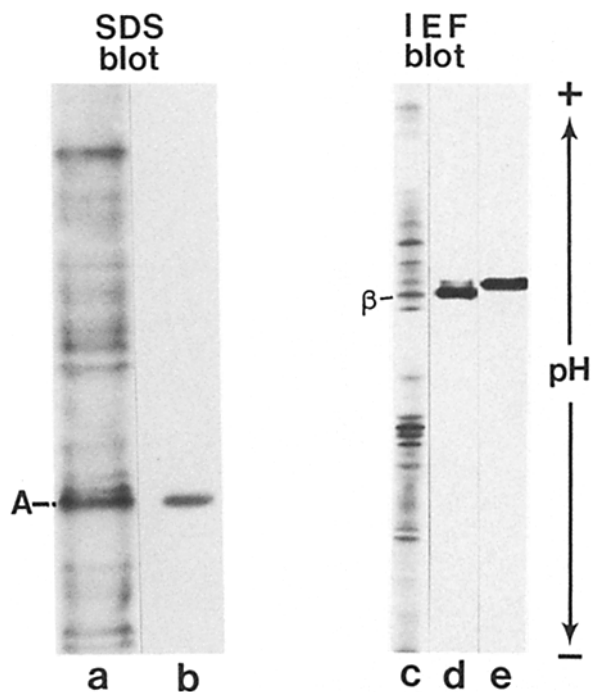


Figure 7. Immunoblot analysis of mouse L cell extracts. Lanes *a* and *b* were electrophoresed on 10% polyacrylamide gels and transferred to nitrocellulose. The immunostained band in lane *b* displayed the same electrophoretic mobility as an actin standard on the same gel (not shown). Lane *c* is a stained IEF gel, and lanes *d* and *e* represent IEF gels transferred to nitrocellulose. Lanes *a* and *c* were stained for protein with Coomassie Brilliant Blue. Lanes *b* and *e* were stained with a 1:5,000 and a 1:12,000 dilution of γ peptide antibody, respectively. Lane *d* was stained with the C4 general actin antibody at a 1:1,000 dilution.

cells stained by double-indirect immunofluorescence with the γ peptide antibody and the monoclonal C4 actin antibody. In Fig. 8, the pairs *c* and *d*, *f* and *g*, and *h* and *i* show the distribution of γ actin versus that of total actin in L cells. Differential actin distributions are not apparent in the comparisons shown. Each brush-like structure or stress fiber observed in *c*, *f*, and *h* can be detected in the images in *d*, *g*, and *i*; and the converse is true. Likewise, in thousands of cells double-stained with γ peptide antibody and C4 monoclonal actin antibody, the distributions appeared to be identical.

It is well documented that the organizational state of actin undergoes changes as rounded-up cells adhere to a substrate and become progressively flattened (10, 19). We wished to determine if the γ isoform could be observed in the transitory actin-containing structures, such as membrane ruffles, which are formed during the course of cell spreading, and if the γ isoform might have a distribution different from that of total actin during the spreading process. As shown in Fig. 9, *a* and *b*, when L cells were stained by indirect immunofluorescence 20–40 min after replating, the γ peptide antibody stained the ruffled edges of the cells very brightly. Small microfilament bundles were detected in a few cells as early as 20 min after replating, and were visible in most cells by 1 h (Fig. 9*c*). The brush-like bundles became larger and more complex within 2–4 h after replating (Fig. 9, *d* and *e*), and by 6 h most cells contained large, brightly stained bundles (Fig. 9*f*). In double-indirect immunofluorescence experiments, the distributions

of γ actin (Fig. 9*g*) and total actin (Fig. 9*h*) were identical in most of the spreading cells. However, at 4–6 h after replating, ~10% of the cells contained microfilament bundles that stained very brightly with the C4 general actin antibody (Fig. 9*j*) but showed little or no staining with the γ peptide antibody (Fig. 9*i*), though other brush-like structures in the same field of cells showed similar patterns of staining with the two antibodies. These results indicate that although the γ isoform is present and thus theoretically able to participate in every organizational state of actin which is observed during cell spreading, localized concentrations of the β isoform may occur in some cells during this process.

Discussion

This report describes the production and use of antibodies specifically reactive with the NH₂ terminus of the γ isoform of nonmuscle actin. Our results confirm the usefulness of the site-directed antibody technique for the study of protein isoforms. One advantage of this approach is that discrete domains of proteins are presented as antigens, so that the resultant antibodies can be used to probe the function of a specific region of the molecule. In the case of actins, the NH₂-terminal segment is of special interest because it is the sequence with the greatest variation among isoforms, and because of the reported binding of myosin subfragments to this domain (39). For this reason, the γ actin peptide antibody we have prepared may be useful in studying the role of the NH₂ terminus in the association of actin with myosin or any of the proteins known to bind to actin (for a comprehensive review of actin-binding proteins, see reference 34).

In terms of general applicability, this method has several advantages over other techniques that have been used for the production of isoform-specific actin antibodies. In one study, Pardo et al. (30) obtained an antibody specific for the smooth muscle γ isoform of actin through the use of a subtractive method, starting from a mixed population of actin antibodies. Their strategy was to prepare a polyclonal antiserum to chicken gizzard actin (which contains predominantly the smooth muscle γ isoform [44]) and to remove the antibodies reactive with all actin isoforms by affinity chromatography with skeletal muscle α actin. The remaining antibodies reacted specifically with γ actin. A disadvantage associated with this method is that actin is a highly conserved protein with limited immunogenicity, so that anti-actin antisera typically are of very low titer (26). Therefore, any method involving fractionation of such an antiserum is likely to produce only very small amounts of isoform-specific antibody. In contrast, with the peptide antibody technique, isoform-specific antisera are easily obtained and these antisera exhibit strong reactivity against actin (usual ELISA titers of >1:20,000). The peptide antibody technique has further advantages; pre-immune sera obtained from the same rabbits which are subsequently immunized with peptide, or sera pre-incubated with the peptide used as the antigen, provide valuable controls for immunofluorescence and immunoblot experiments. These controls are neither available nor valid with affinity purification methods.

The monoclonal antibody technique has also been applied to the production of isoform-specific actin antibodies: to date, monoclonals selective for all muscle isoforms of actin (22) and for smooth muscle isoforms of actin (13) have been

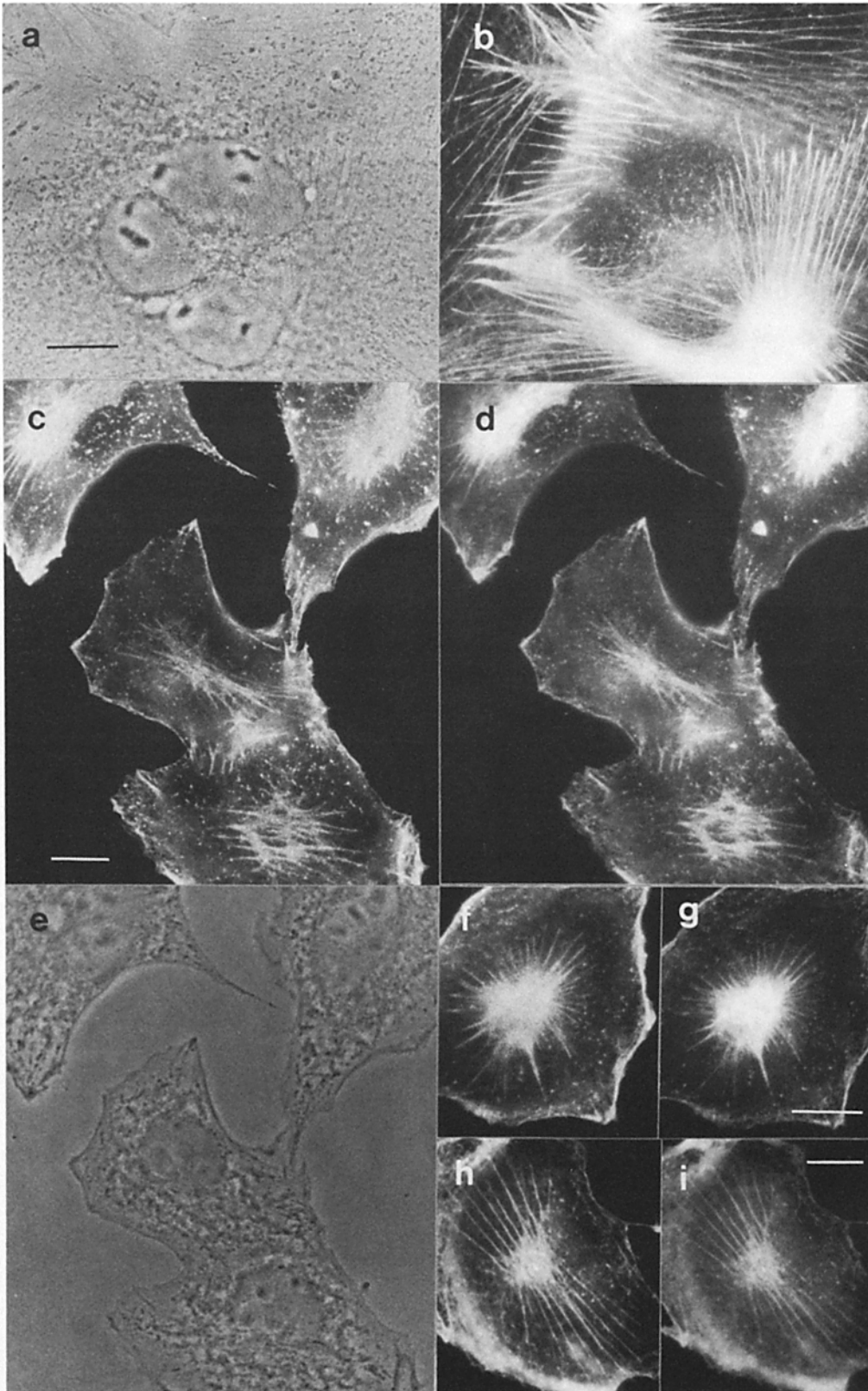


Figure 8. Indirect immunofluorescence localization of actin isoforms in mouse L cells. (a and b) Phase-contrast and corresponding fluorescence image of a giant L cell, stained with γ peptide antibody (1:12,000 dilution) followed by a rhodamine-conjugated goat anti-rabbit IgG. (c-i) Simultaneous immunolocalization of γ actin and total cellular actin in mouse L cells. Indirect immunofluorescent staining of methanol-fixed L cells with (c, f, and h) γ peptide antibody (1:12,000 dilution) followed by a fluorescein-conjugated goat anti-rabbit IgG; and (d, g, and i) monoclonal C4 actin antibody (1:60 dilution) followed by a rhodamine-conjugated goat anti-mouse IgG. (e) Phase-contrast image of cells shown in c and d. Bars, 10 μ m.

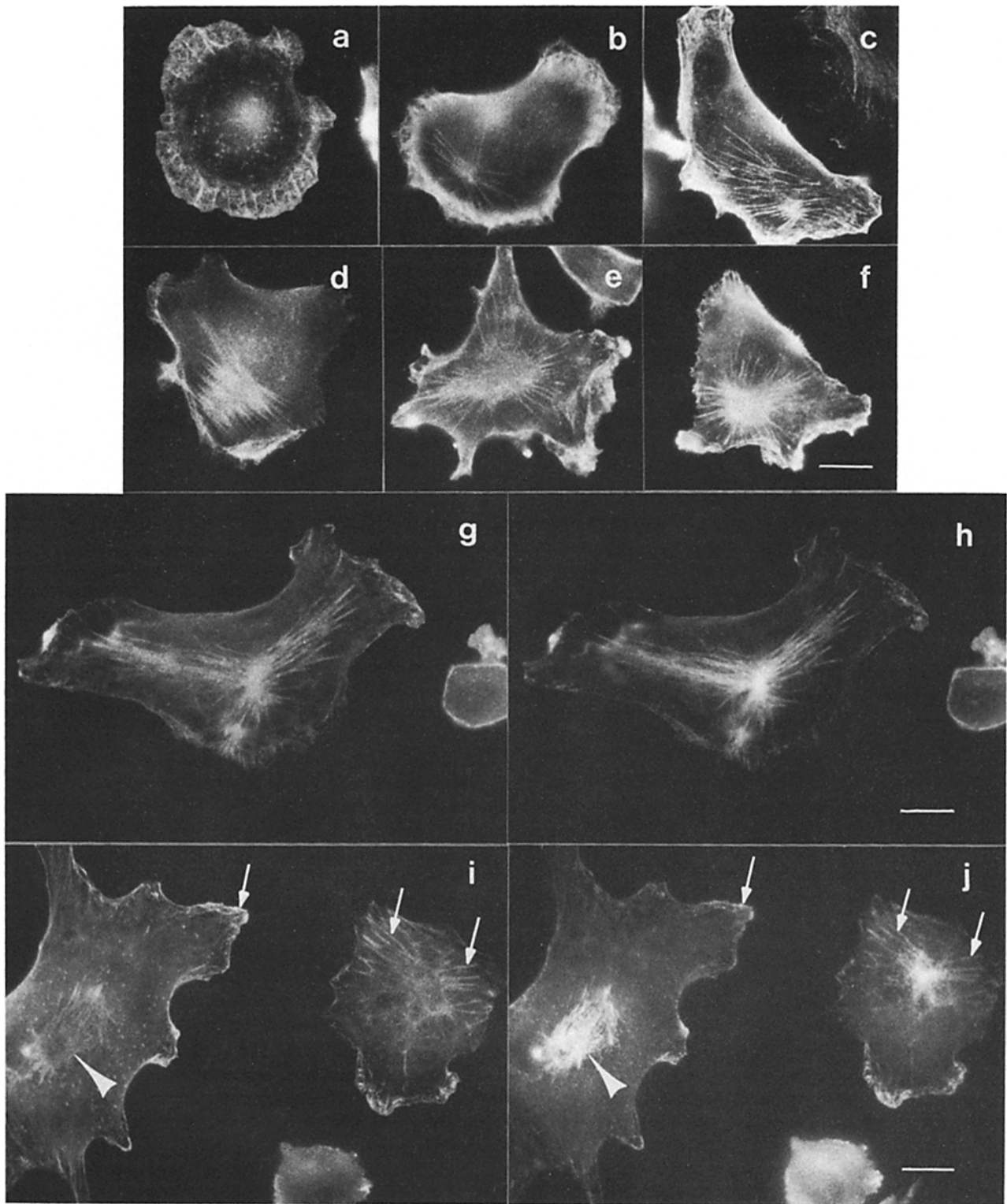


Figure 9. Immunostaining of actin isoforms in spreading L cells. Trypsinized cells were replated onto glass coverslips, then methanol fixed after (a) 20 min, (b) 40 min, (c) 1 h, (d) 2 h, (e) 4 h, or (f-j) 6 h. a-g and i were stained with the γ peptide antibody at a dilution of 1:12,000; h and j were stained with the C4 antibody at a dilution of 1:60. The pair in g and h shows the staining pattern most commonly observed; here, the distribution of γ actin (g) and total actin (h) appear to be identical. The pattern seen in the pair in i and j was found in 10% of cells at 4 or 6 h after replating. Small arrows indicate regions where the staining with the γ peptide antibody (i) was at least as bright as with the C4 antibody (j) and large arrowheads indicate regions in which, in contrast to the C4 antibody, the γ peptide antibody stained dimly or not at all.

generated. However, one problem associated with this method is that the low immunogenicity of actin, combined with the high degree of homology between isoforms, greatly reduces the likelihood that hybridomas secreting an isoform-specific antibody will be isolated. Other problems encountered with isoform-specific monoclonal antibodies include the possible low selectivity or low affinity of the antibodies. In addition, when an actin monoclonal antibody is obtained, it is frequently a difficult process to determine the epitope at which the antibody binds (35). It is an obvious advantage to know the domain at which the antibody binds in order to predict cross-reactivity with other proteins, or to study the biochemistry of functional domains. Our current results as well as those previously reported (5) demonstrate the ease with which antibodies reactive with a pre-selected domain of a desired actin isoform can be elicited by immunization with a peptide. Using this technique, it should be possible to produce antibodies that can distinguish between any two actin isoforms by preparing peptides that correspond to the differing regions of the two actin sequences. Cross-reactivity with a similar sequence contained in another of the tissue-specific actin isoforms is not a problem as long as the cross-reactive species is not expressed by the cells or tissue of interest.

We used our specific peptide antibody to localize γ actin in cultured cells by immunofluorescence. One cell type chosen for these experiments was the mouse L line, in which Sakiyama et al. (33) previously reported that no expression of γ actin was detectable by the technique of two-dimensional gel electrophoresis. However, in Western blot experiments, we found that these cells do contain γ actin. We also found in indirect immunofluorescence experiments that all L cells express γ actin; every cell contains brush-like microfilament bundles which stain brightly with the γ actin peptide antibody. Thus, this antibody can be used for detection of nonmuscle γ actin in extracts and in immunofluorescence localization even in cells and tissues in which this isoform is a minor component. This is similar to a previous study in which an α -specific peptide antibody was used to detect α actin in myogenic cultures which contained a vast excess (50–100-fold excess over the α isoform) of β and γ actins (5).

It was also of interest to determine if an isoform-specific distribution of β and γ actins could be detected at the level of light microscopy. Actin isoforms are the products of different genes, and they are present in amounts which vary among cell types (7, 9, 42). They have been speculated to differ in their *in vivo* function and/or subcellular localizations (11, 17, 30, 32, 40, 43). One might also expect that a minor protein form, such as γ actin in L cells, might be present in a minority of cells or could be sequestered in minor cellular structures. However, our results suggest that neither is the case. In the four types of well-spread cells that we have examined, all stress fibers visible by phase-contrast microscopy in all cells were found to contain detectable γ actin. In addition, all microfilament bundles observed which stained with C4, the monoclonal antibody which reacts with all actin isoforms, also stained with our γ peptide antibody. Furthermore, there was no evidence of an isoform-specific localization in spreading L cells. Although a minority of the spreading cells contained regions which appeared to be more brightly stained with C4 than with the γ peptide antibody, no actin-containing structures devoid of γ actin were found.

These results are different from those obtained by Pardo et al. (30) with their affinity-purified γ actin antibody. In sections of mouse diaphragm stained by immunofluorescence, γ actin was found to co-localize with mitochondria and was not detected in the myofibrillar cross-striations, which were found to contain only α actin. It is not clear from their results, however, whether the different staining patterns might not be due to inadequate concentrations of the γ actin antibody. We have found that very dilute antibodies can be used in immunofluorescence staining only if the volume of antibody solution is fairly large (≥ 10 ml). When small volumes are used, as they were by Pardo et al. (30), the antibody concentration must be quite high, typically 10–200 times higher than that used to stain Western blots. Also, the antibody must have a high affinity for its antigen, which affinity-purified antibodies might not have. The mitochondria-associated staining pattern which Pardo et al. (30) observed was also found to be highly sensitive to the type of fixation that was used; that is, it was only visualized in methanol-fixed sections. In contrast, we consistently found the same pattern of stress-fiber staining in methanol-fixed cells (which were used most frequently in experiments) and in cells fixed with either formaldehyde or glutaraldehyde and extracted with acetone. However, although Pardo et al. (30) showed reactivity of their antibody with actin in extracts of nonmuscle cells (mouse lymphocytes), they did not use tissue culture cells in any of their experiments. The actin antibody of Pardo et al. (30) may be staining minor structures which are found in mouse diaphragm but not in the cultured cells that we have examined. For all these reasons, it is not possible to make a direct comparison between our findings and theirs.

Aside from studying the sorting of nonmuscle γ actin, the possibility that other isoforms are sorted has also been examined. Hall et al. (15) and Lubit and Schwartz (24) have used a specific anti-actin antibody to determine that nonmuscle actin was present in membrane systems but not in sarcomeres of muscle cells. Herman and D'Amore (16) also used antibodies selective for nonmuscle and for muscle actins to detect these forms of actin in cultured cells and in cryosectioned tissues, but they were unable to determine whether isoform segregation occurred. In any case, both of these groups localized nonmuscle versus muscle isoforms in differentiated cells and tissues; it is possible that these isoforms possess a capacity for subcellular sorting which nonmuscle β and γ isoforms do not.

Our findings are in agreement with those of McKenna et al. (25) who injected fibroblasts with a mixture of fluorescently labeled actin isoforms and found no sorting of the muscle and nonmuscle forms; both were incorporated into the same stress fiber bundles. If cells are capable of actin sorting at all, then one might expect nonmuscle cells to distinguish between nonmuscle and muscle actins, as they are more different from one another than are the two nonmuscle isoactins, yet in these experiments no sorting of these was observed. The possibility remains that sorting of actin isoforms occurs cotranslationally, so that injected actins are excluded from the sorting process. However, our results indicate that endogenous nonmuscle actin isoforms are not segregated into different stress fibers.

To date, we have found no evidence of an isoform-specific subcellular localization of nonmuscle γ actin in cells which

contain only the two nonmuscle actins. These results represent the simplest case, and they do not rule out the possibility of sorting in cells which express more than two actin isoforms or contain pairs of isoforms other than β and γ . Moreover, there are several patterns of isoform-specific localization which we would be unable to detect using our γ peptide antibody in light microscope level immunolocalization. For example, since stress fibers composed entirely of γ actin would stain with both the C4 antibody and the γ peptide antibody, this pattern of isoform segregation would not be detected by comparing the two immunofluorescence images. However, any stress fibers composed of the β isoform alone would have been easily detected in such a comparison, as they would stain only with the C4 antibody. Hence, we conclude that there are no stress fibers in the cell types we examined that contain only β actin. If β and γ actin are differentially localized, it is possible that the two isoforms are sorted into different microfilaments but that each stress fiber contains both types of microfilaments. Alternatively, there may be a regional or periodic distribution of actin isoforms along individual microfilaments. Perhaps these or other instances of isoform-specific subcellular localization will be found in immunoelectron microscopy. Our cell-spreading result, in which a few cells contained microfilament bundles that did not react strongly with the γ peptide antibody, suggests to us that at the ultrastructural level some interesting results will be obtained. Also, we may find differences in actin localization in other types of cells, such as neuronal cells, muscle cells, sperm, or sections of tissues.

Even if the distribution of actin isoforms within microfilaments is eventually found to be random, this by no means rules out the possibility of functional differences between β and γ actins. It may be that the many actin-binding proteins found in nonmuscle cells interact preferentially with one or the other isoform, so that the presence of both β and γ isoforms of actin within a microfilament allows each filament to perform the full range of functions which are associated with actin in nonmuscle cells.

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