Identification of New Actin-associated Polypeptides That Are Modified by Viral Transformation and Changes in Cell Shape

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Abstract. By using a monoclonal antibody we have identified a new polypeptide doublet (C4^h and C4^l) of M_r ~21 kD and pI 8 and 7, respectively, that is associated with and (at the immunofluorescence level) uniformly distributed on actin filament bundles in rat, mouse, and other vertebrate species. C4 is absent in neurones, erythrocytes, and skeletal muscle but the epitope is evolutionarily conserved as it is present in invertebrates such as molluscs and crustaceans.

C4^h is not found in cells such as lymphocytes and oncogenically transformed mesenchymal cells where actin stress fiber bundles are reduced in number or absent. C4^l, on the other hand, is always present. C4^h expression can also be blocked by switching normal nontransformed mesenchymal cells from adherent to suspension culture. Reexpression of C4^h occurs 24 h after these cells are returned to normal adherent culture conditions, but can be blocked by either actinomycin D or cycloheximide, suggesting that the expression of this epitope is regulated at the transcriptional level.

IN normal mesenchymal cells, grown in dissociated cell culture, actin is often organized into large bundles of stress fibers that traverse the cytoplasm (43, 55). Stress fibers (10) are associated with anchorage dependence, reduced mobility, and contact inhibition (25, 33). Transformation of mesenchymal cells by chemical carcinogens or oncogenic nucleic acids causes a loss of stress fiber bundles with a concomitant alteration in cell shape, loss of contact inhibition, and enhanced tumor-forming potential (1, 7, 19, 27, 31, 32, 42, 43, 52, 55). Transformation also causes the loss of the α actin isoform (32), decreased actin and tubulin synthesis (15), and the production in a fibroblast line of a variant actin form (31). Many proteins are associated with and modify the physical state of actin in nonmuscle cells, thereby playing a crucial part in various actin-based cell movements (44, 45, 56). So far, little is known about the biochemical effects of oncogenic transformation on these actin-associated proteins except that tropomyosin isoforms are modified (38), the phosphorylation of vinculin increased eightfold (47), and the cellular level of caldesmon was reduced by two-thirds (41). Culture conditions that cause changes in cell shape are also known to affect the cytoskeleton (for a review see reference 3) but of the many actin-associated proteins only the biochemical expression of vinculin has so far been investigated (54). In this study we have used a monoclonal antibody that labels actin stress fiber bundles to identify a new actin-associated polypeptide doublet (C4^h and C4^l), the expression of which is biochemically modified not only by oncogenic transformation with either DNA or RNA tumor viruses but also, in a reversible manner, by changes in cell shape. C4 is found in all vertebrate species so far examined. It is expressed by smooth, but not skeletal muscle cells, and a variety of nonmuscle cells apart from neurones and red blood cells.

Materials and Methods

Immunogen/Hybridoma Production

The monoclonal antibody used in these studies (anti-C4) was produced by a hybridoma from a fusion that has been previously described (28). The class of anti-C4 was found to be IgG$_1$ by Ouchterlony immunodiffusion using rabbit anti-mouse class-specific antisera (Miles Laboratories, Inc., Elkhart, IN) to IgA, IgG1, IgG2a, IgG2b, IgG3, and IgM. Ascites were raised by injecting nu/nu mice with 1 × 10^7 cells and harvesting the ascitic fluid 10 d later.

Purification of Monoclonal Antibody

Supernatant (or ascites) was adjusted to pH 8.6, passed over a Sepharose-protein A column (Pharmacia Inc., Piscataway, NJ) eluted at pH 5, dialyzed into PBS, aliquoted, and used at a concentration of 50 µg/ml.

Polyclonal Antibody against Vimentin

An IgG fraction of rabbit anti-vimentin (22) was purified on DEAE as described (28) and used at a concentration of 60 µg/ml.

Adherent Cell Culture

3T3, SV-40 3T3, and human epithelial cells (Detroit 98) were adjusted to ~10^4 cells/ml in DME plus 10% heat-inactivated FCS, plated in 1-ml ali-

quots on to 13-mm-diam coverslips in 24-well Linbro plates (Flow Laboratories, Hamden, CT) or Falcon Labware flasks (Oxnard, CA), and used for immunofluorescence or biochemistry 24–72 h later. Secondary cultures of rat embryo fibroblasts (REF)[1] were obtained by trypsinizing the dissected limbs of 15-d-old embryos and culturing as above.

Suspension Cell Culture

Cells ($\sim$10^6/ml REF) were plated on petri dishes (Falcon Labware) that had been smeared with a layer of high vacuum grease (Beckman Instruments, Inc., Palo Alto, CA or Dow Corning Corp., Midland, MI). This was nontoxic with 97–100% of cells reattaching and spreading normally after 72 h in suspension culture. Cycloheximide and actinomycin D (Sigma Chemical Co., St. Louis, MO) were used at concentrations of 25 and 2.5 µg/ml, respectively (11, 17). At these concentrations adherent cell viability (measured by trypan blue exclusion) was >95% with both reagents.

Immunofluorescence

Cells were permeabilized by (a) formaldehyde fixation (3.5% for 12 min at room temperature) followed by cold acetone, (b) cold methanol alone, or (c) 0.1% Triton X-100 in buffer A (50 mM KCl, 4 mM MgCl$_2$, 1 mM EGTA, 10 mM imidazole pH 7, 1 mM NaN$_3$ [35]) or buffer B (as buffer A except for 100 mM KCl) for 5 min at 4°C. Subsequent processing, reagents, and immunofluorescence microscopy were as previously described (28, 29) except that an IgG-specific goat anti–mouse rhodamine (Cappel Laboratories, Inc., Cochranville, PA) at a dilution of 1:100 was used to label anti-C4. Fluorescein-phalloidin (Molecular Probes, Inc., Junction City, OR) was used at a concentration of 3 µM. Cytochalasin D (Sigma Chemical Co.) and colchicine (Sigma Chemical Co.) were used as described (28). For microinjection, anti-C4 was dialyzed into injection buffer and injected as described (24) at a concentration of 20 mg/ml.

Biochemistry

SDS-PAGE/Immunoblotting. $\sim$10^6 cells (or the equivalent amount of tissue) were solubilized, the total lysate run on either 5–15% gradient or 12% SDS-PAGE using the method of Laemmli (26), and immunoblotted onto nitrocellulose, all as described (28) except that protein A–purified anti-C4 was used at a concentration of 50 µg/ml. Dye-coupled molecular mass markers (Bethesda Research Laboratories, Gaithersberg, MD) were blotted in parallel and then cut off with pinking shears to facilitate realignment after drying (28). 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 µg/ml soy bean trypsin inhibitor (Sigma Chemical Co.), and 5 mM EDTA were routinely added at the lysis stage. Additional proteolysis inhibitors used (all from Sigma Chemical Co.) were 10 µg/ml chymostatin, 2 µg/ml aprotonin, 0.1 mM leupeptin, 10 µg/ml TLCK, and 5 mM iodoacetamide. Sheep aorta smooth muscle F actin thin filament preparations, purified F actin filaments, and crude aorta (from Dr. S. Marston) (34, 51) were processed as above. Bovine brain calmodulin (Sigma Chemical Co.) (9), gizzard light chain myosin (from Dr. J. Kendrick-Jones) (23), chick embryo brain actin-depolymerizing factor (from Dr. J. Bamburg) (2), cofilin (from Dr. E. Nishida) (40), and 3T3 cell N-ras P21 (from Dr. C. Marshall) (53) were solubilized as above and loaded at concentrations of 5, 5, 2, 2, and 5 µg per slot, respectively. REF were used as a positive control in each instance.

Affinity Column Purification of C4. 10 mg of monoclonal anti-C4 were coupled to Affigel 10 (Bio-Rad Laboratories, Richmond, CA). Cell samples ($\sim$5 × 10^8 REF) were trypsinized off Falcon Labware flasks, washed in PBS, lysed for 25 min at 0°C in 15 ml of 50 mm Tris pH 7, 0.5% Triton X-100, 1 µg/ml soybean trypsin inhibitor, 1 mM PMSF, 100 µM leupeptin, 2 mM EDTA, 5 mM NaN$_3$, and spun at 20,000 g for 30 min to remove insoluble debris. Supernatants were passed over the column at $\sim$5 ml/h, the bound fraction eluted with 50 mM diethylamine pH 11.5, immediately neutralized with 1 M Tris pH 7, dialyzed into 50 mM Tris pH 7, and then concentrated to $\sim$1 ml in Aquacide 1 (M_r 70 kD; Calbiochem-Behring Corp., La Jolla, CA). Sample purity was assayed by SDS-PAGE immunoblotting followed by either amido black staining to visualize total protein or anti-C4 antibody/Rab anti-mIg peroxidase (DAKOPATTS, Copenhagen).

Two-dimensional Gel Electrophoresis. NEPHGE gels loaded with $\sim$5 µg of purified C4 were subjected to electrophoresis for 1,600 Vh, then run on second dimension 12% SDS-PAGE, and stained with silver nitrate as de-

scribed (30). These conditions generated pI gradients between 4.2 and 9.2 which were measured using blank gels run in parallel.

Phosphatase Digestion. REF were solubilized, run on 12% SDS-PAGE, and immunoblotted as above. Nitrocellulose strips were then incubated with either 70 U of alkaline phosphatase (type XXXI; Sigma Chemical Co.) in 0.1 M Tris pH 5, plus 2 mM PMSF or buffer alone for 16 h at 37°C (16). Strips were then labeled with anti-C4 as described above or stained with amido black to visualize total protein (16).

In Vivo Metabolic Labeling/Immunoprecipitation/SDS-PAGE. 5 × 10^4 REF/ml were plated overnight in Falcon Labware flasks, rinsed in DME (minus methionine) plus 2% FCS, and incubated in the same medium for 30 min. Fresh medium (3.3 ml) containing 0.5 mCi/ml of [^{35}S]methionine (SJ 204; Amersham International, Amersham, UK) was added, the cells incubated for 4 h, rinsed in DME (minus methionine) for 30 s, and either chased for 48 h in complete DME plus 10% FCS or trypsinized immediately, washed in DME plus 10% FCS, and once in 20 ml of PBS. Cell pellets were then lysed by resuspending them for 10 min at 0°C in 100 µl of lysis buffer (10 mM Tris pH 7.5, 2 mM EDTA, 1 mM PMSF, 0.1 mM leupeptin, 1 µg/ml soy bean trypsin inhibitor, and 0.5% Triton X-100), centrifuged at 10,000 g for 10 min at 4°C. The supernatant was then removed and precleared by adding 10 µg of normal mouse IgG (prepared by DEAE fractionation) for 1 h at 4°C followed by 50 µg of affinity-purified rabbit anti–mouse IgG for 2 h at 4°C, and then centrifuged as above. The precleared supernatants were incubated with preformed immune complexes overnight at 4°C (immune complexes were formed by cross-linking 10 µg of either monoclonal anti-C4 or an IgG monoclonal anti-β tubulin [Amersham International] with 50 µg of affinity-purified rabbit anti–mouse IgG [in a total volume of 45 µl] for 4 h at 4°C, and then washed twice by centrifugation [as above] in lysis buffer before adding to supernatants). Samples were then centrifuged as above and the pellets washed by centrifugation: twice in lysis buffer, once in lysis buffer plus 0.5 M NaCl, and once in lysis buffer alone. Pellets were solubilized in Laemmli sample buffer and $\sim$3.5 × 10^4 counts per slot loaded on to 12–15% gradient gels, which were fluorographed with Amplify (Amersham International), dried, and exposed to Kodak X-Omat AR film. These experiments were also carried out using 1 mCi/ml [^{3}H]leucine and [^{3}H]lysine (TRK683 and TRK752; Amersham International). The position of the C4 doublet was determined by running a parallel track of affinity-purified C4^h and C4^l from REF, the gel was then lightly stained with Coomassie Blue, dried, and the two bands marked with radioactive ink before exposure on film as above. For immunoblotting of C4 immune complexes samples were loaded on to 12–15% gradient gels, transferred to nitrocellulose, labeled as described in the previous section, and the ratio of C4^h/C4^l was obtained by excising these bands from nitrocellulose and counting the strips in a gamma counter.

Results

Immunofluorescence

Double immunofluorescence studies using fluorescein-coupled phalloidin together with the monoclonal antibody (anti-C4) showed that both uniformly stained linear actin filament stress fiber bundles in all cells containing these structures that we examined (Fig. 1, a and b). In no instance did we find any area of stress fiber bundles labeled with phalloidin but not with anti-C4 (Fig. 1, a and b). Neither cell processes nor the diffuse actin network which is also present in these cells (30) were appreciably stained with anti-C4 (Figs. 1, a and g). Additional evidence that anti-C4 stained stress fibers was provided by the disruption of the labeled fibers in cytochalasin D–treated cells (Figs. 1, c and d). In colchicine-treated cells (Fig. 1 e), where the intermediate filament network has collapsed, no such disruption of antibody labeling occurs (Fig. 1 f). When cells were microinjected with anti-C4, permeabilized with methanol, and then labeled with rhodamine-coupled anti–mouse IgG the labeling pattern was identical to that observed in prefixed cells (Fig. 1 a), indicating that the distribution of the antigen on stress fibers is the native distribution of this protein (Fig. 1 g). In contrast, cells where stress fibers are reduced in numbers or absent (such as lym-

Figure 1. Immunofluorescence localization in REF of C4. Double immunofluorescence showing superposition of anti-C4 (*a*) and fluorescein–phalloidin (*b*) in control cells and anti-C4 (*c*) and fluorescein–phalloidin (*d*) in cytochalasin D–treated cells. Anti-C4 staining is unaffected in colchicine-treated cells (*e*) where intermediate filaments (labeled with rabbit anti-vimentin) have collapsed around the nucleus (*f*). Note the linear stress fiber distribution of microinjected anti-C4 (*g*). Noninjected cells are unlabeled (*asterisk*). Mainly, diffuse non-linear cytoplasmic staining is present in epithelial cells (*h*). C4 is retained in ∼50% of cells when they are detergent extracted in buffer A plus 20% ethanediol (*i*) but is lost when ethanediol is removed (*j*). Bars: (*a–g*) 20 μm; (*h–j*) 10 μm.
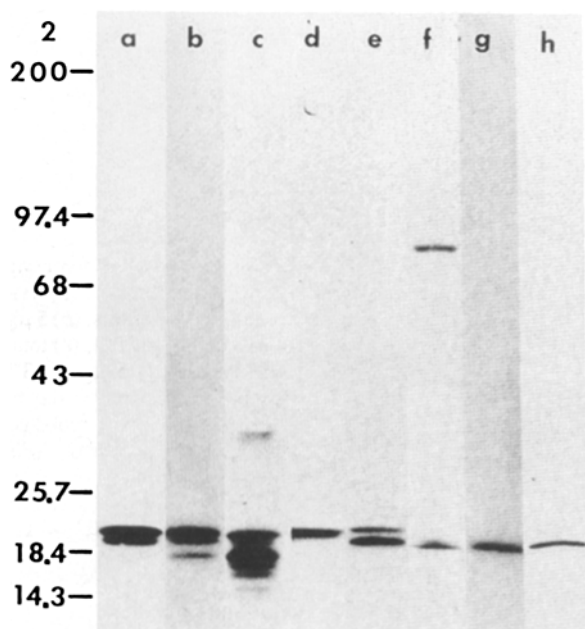
Figure 2. Immunoblot analysis of C4 in different cell and tissue types. C4 is present as a doublet in all normal mesenchymal cells such as rat fibroblasts (*a*). No variation was found in the relative molecular mass of C4^l between different tissue within the same species (rat): fibroblasts (*a*), smooth muscle (*b*), heart (*f*), and brain (*g*). However, 2–3-kD variations in the relative molecular mass of C4^l were found when the same tissue from different vertebrate species was compared: rat (*b*), chicken gizzard (*c*), fish (*d*), and frog (*e*). C4^h is absent in heart where an additional band at 79 kD is present (*f*), in brain (*g*), and in epithelial cells (in this instance Detroit 98—a human cell line) (*h*). The following tracks were run in parallel but on separate gels: *b*, *f*, and *g*; and *c–e*.

ence of 2–3 kD in the relative molecular masses of C4^l between vertebrate species when the same tissue (smooth muscle) was examined (Fig. 2, *b*, *c*, *d*, and *e*). C4^h (not C4^l) is absent in heart (where an additional band of M_r 79 kD is always present; Fig. 2*f*), brain (Fig. 2 *g*), epithelial cells (Fig. 2 *h*), and lymphocytes (not shown). C4^h has not, so far, been found in any cell or tissue type without the concomitant expression of C4^l. C4 is absent in neurones, erythrocytes, and skeletal muscle but is present in invertebrates such as molluscs and crustaceans (not shown). Faint staining of bands at M_r 180, 39, and 31 kD was observed in some experiments regardless of cell type (Figs. 2 *c* and 5, *g–k*). The doublet formation of C4 was unaffected by (*a*) the presence or absence of high concentrations of reducing agents (20% 2-mercaptoethanol or 100 mM dithiothreitol) and (*b*) a spectrum of proteolysis inhibitors.

Biochemical evidence for the association of C4 with actin filaments was obtained by immunoblot analysis of sheep aorta (Fig. 3, *a* and *b*) and aortic smooth muscle actin thin filaments. These are standard preparations of isolated F actin filaments to which several actin-associated proteins are bound (34–37, 39, 46, 51) and were strongly recognized by anti-C4 antibody (Fig. 3, *c* and *d*). In contrast purified F actin filaments, prepared by removing these associated proteins from thin filaments (by increasing ionic strength and removing ethanediol [34]), were completely unlabeled by anti-C4 (Fig. 3, *e* and *f*). Control tracks of fibroblasts labeled with anti-C4
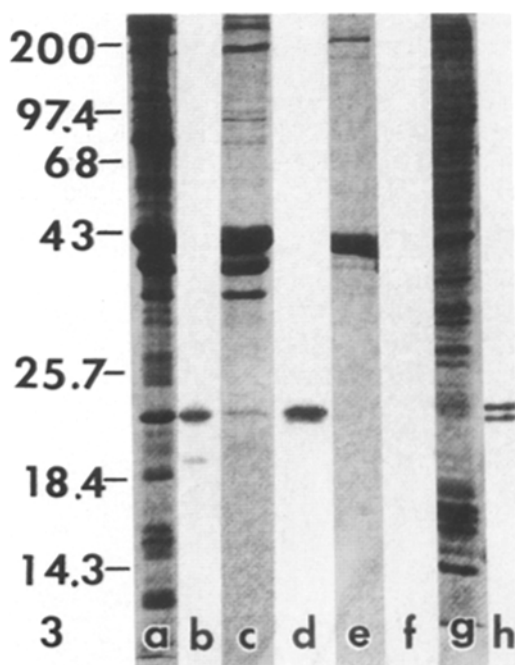
phocytes, dividing or transformed mesenchymal cells, and epithelial cells [Fig. 1 *h*]) were stained diffusely throughout their cytoplasm by the monoclonal antibody. Permeabilization of fibroblasts for 5 min in buffer B (with 100 mM KCl) plus 1% Triton X-100 and 20% ethanediol eliminated most antibody labeling at the immunofluorescence level (not shown). In contrast, the level of C4 staining was not appreciably reduced in ~50% of cells detergent extracted for 5 min in buffer A (with 50 mM KCl) plus ~1% Triton X-100 and 20% ethanediol (34, 36) (Fig. 1 *i*). Removal of ethanediol from buffer A resulted in the almost total loss of C4 staining (Fig. 1 *j*).

Biochemistry

SDS-PAGE coupled with immunoblot analysis of total cell proteins show that the monoclonal antibody recognizes a closely spaced doublet of two polypeptides (C4^h and C4^l) with an M_r of 21 kD. These polypeptides are usually present in equal amounts in normal fibroblasts (occasionally with C4^h in excess of C4^l) and are separated by 0.5–1 kD in these cells (Fig. 2 *a*) and smooth muscle (Fig. 2 *b*). When different tissues within the same species (rat) were run on SDS-PAGE and then transferred to nitrocellulose no significant variation was found in the relative molecular mass of C4^l (Fig. 2, *a*, *b*, *f*, and *g*). However, there is a differ-

Figure 3. Immunoblot analysis of sheep aorta and actin filament fractions. C4 is present as a doublet (with C4^h in excess of C4^l in this experiment) when total aorta (*a*) is transferred to nitrocellulose (*b*). Identical results were found when actin thin filaments (*c*) purified from aorta (these preparations contain F actin filaments to which are bound several actin-associated proteins [35]) were transferred to nitrocellulose (*d*). Removal of these associated proteins to leave F actin filaments (*e*) paralleled the complete loss of anti-C4 label (*f*). Control tracks of 3T3 cells are seen in *g* and *h*. Tracks *a*, *b*, and *c–h* are from the same gels.

showing the presence of a doublet similar to Fig. 3 *b* and *d* are shown in Fig. 3 *g* and *h*.

By affinity column purification on anti-C4 conjugated to Affigel, ~1.5 µg SD ± 0.7 of C4 was obtained from 10^6 REF, with the recovery of $C4^h$ greater than $C4^l$ in these experimental conditions (Fig. 4, *a* and *b*). NEPHGE/SDS-PAGE showed that purified $C4^h$ and $C4^l$ have 8 and 7 pI, respectively (Fig. 4 *c*). Neither $C4^h$ nor $C4^l$ have so far been found to be phosphorylated by ^{32}P incorporation, and alkaline phosphatase digestion of immunoblots of REF did not modify the characteristics of the epitopes (not shown). Immunoblot analysis showed that anti-C4 did not recognize other proteins of similar molecular mass that are associated with actin, namely cofilin (3), actin-depolymerizing factor (2), chicken gizzard light chain myosin (23), clamodulin (9), and 3T3 cell N-ras P21 (53) (Fig. 5, *b* and *d*). In these experiments also, an REF control was transferred in parallel and was always labeled strongly by anti-C4 (Fig. 5, *c* and *e*).

In vivo labeling of REF with either [^{35}S]methionine or [^{3}H]leucine and [^{3}H]lysine, followed by Triton X-100 extraction, and immunoprecipitation with anti-C4 showed that two bands at M_r 21 kD were coprecipitated (Fig. 6 *b*). The top band ($C4^h$) had a two- to fourfold greater incorporation of radioactivity than $C4^l$ after both a 4-h pulse (Fig. 6 *b*) and a 48-h chase (Fig. 6 *c*). This ratio was also found when immunoprecipitates were transferred to nitrocellulose and probed with anti-C4/anti-^{125}I-mIgGt, thus the higher incorporation of label in $C4^h$ compared with $C4^l$ may reflect the amount of antigen present in the two bands (not shown). A band at 18 kD was found in these experiments (Fig. 6, *b* and *c*) but not when cell lysates were directly solubilized in SDS before immunoblotting (compare Figs. 2 *a* and 7, *a–k* with Fig. 6, *b* and *c*). Neither $C4^h$ nor $C4^l$ were seen (even after 8–10 d autoradiography at −70°C) when adherent cells, pulse labeled for 4 h with [^{35}S]methionine were cultured in suspension for 72 h, and then immunoprecipitated with anti-
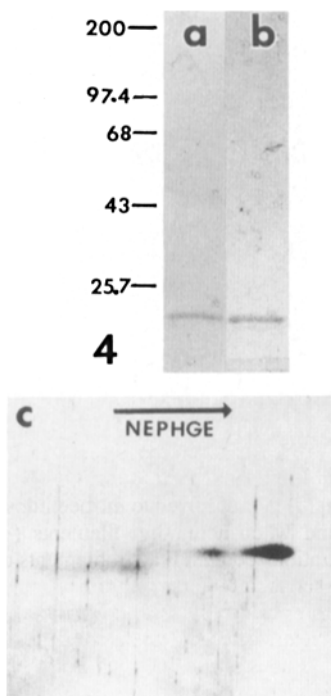
Figure 4. Affinity column purification of C4 isolated from REF followed by SDS-PAGE/immunoblotting. (*a*) Amido black staining of purified C4 fraction. (*b*) Anti-C4 label of track *a*. (*c*) NEPHGE gel of purified C4 showing a pI of 8 for $C4^h$ and 7 for $C4^l$.
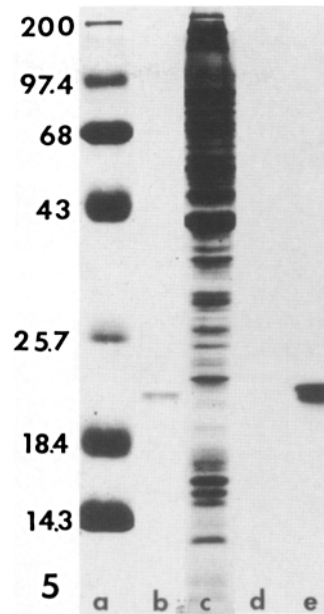

Figure 5. (*a–c*) Coomassie Blue-stained tracks of (*a*) molecular mass markers, (*b*) 5 µg of 3T3 cell N-ras P21, (*c*) total REF lysate. (*d*) 5 µg of 3T3 cell N-ras P21 transferred to nitrocellulose and incubated with anti-C4. No staining is seen in this track. (*e*) Total REF lysate control transferred to nitrocellulose and probed with anti-C4 showing a doublet at M_r 21 kD. All tracks are from the same gel.

C4 (not shown). Both $C4^h$ and $C4^l$ are, however, present in adherent cells after a 72-h chase (not shown). Control experiments using anti-β tubulin showed the presence of one band at ~50 kD with no bands visible at 21 kD (Fig. 6 *d*).

Effect of Oncogenic Transformation and Cell Shape on C4 Expression

When fibroblastic cells such as 3T3 or Rat 1 are transformed with either DNA (SV-40 [43, 55]) or RNA tumor viruses (Rous sarcoma wild type and ts mutant LA 29 [52]) a striking and reproducible change occurs in the biochemical expression of C4. This is seen as a complete loss of the $C4^h$ (never $C4^l$ which is always present) band (Fig. 7, *a* and *c*). A similar effect has been found in transformed human mesenchymal cells (not shown [20]). In all instances the nontransformed cells expressed both $C4^h$ and $C4^l$ (Fig. 7, *b* and *d*). The effect of viral transformation on $C4^h$ can be mimicked by culturing mesenchymal cells in suspension. These cells, when prevented from attaching to the substratum, show a progressive and almost total loss of $C4^h$ over a 72-h period (compare Fig. 7, *e* and *f*). Similar results were obtained when cells were scraped rather than trypsinized off culture dishes before suspension culture (not shown). Reexpression of $C4^h$ did not occur in detectable amounts 8 h after cells grown in suspension culture for 72 h (Fig. 7 *g*) were allowed to reattach and spread (Fig. 7 *h*), but C4 attained its normal level after 24 h in adherent culture (Fig. 7 *i*). However, the reexpression of $C4^h$ was blocked in respreading cells incubated for 24 h with either actinomycin D (Fig. 7 *j*) or cycloheximide (Fig. 7 *k*) after 72 h in suspension culture.

To look for any correlation between the expression of $C4^h$ and the presence of actin stress fiber bundles, cells from the same cultures used for the biochemical experiments above were processed for immunofluorescence and labeled with anti-C4. While after 8 h of respreading (in the absence of inhibitors) only 13% of cells had visible stress fibers (Fig. 8 *a*, and compare with gel in Fig. 7 *h*), after 24 h 72% of cells
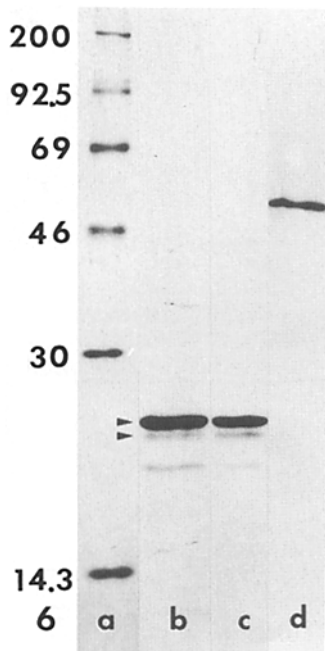
Figure 6. Autoradiograph showing (*a*) molecular mass markers, (*b*) immunoprecipitate of C4 from REF, after a 4-h pulse with [³⁵S]methionine. Two bands (C4^h and C4^l) at M_r 21 kD are present (*arrowheads*) with the upper (C4^h) containing about two to four times as much radioactivity as C4^l. (*c*) Immunoprecipitate of C4 after a 48-h chase. Note that there is no apparent increase in the amount of radioactivity present in C4^l during this period. A band of varying intensity at M_r 18 kD was occasionally found in these experiments. (*d*) Control immunoprecipitate of anti-β tubulin with one band present at ~50 kD. All tracks are from the same gel.

had large numbers of stress fibers traversing the cytoplasm on which the distribution of fluorescent-phalloidin (not shown) paralleled that of anti-C4 (Fig. 8 *b*, and compare with gel in Fig. 7 *i*). In contrast, of the cells respread for 24 h in the presence of either cycloheximide or actinomycin D (Fig. 8 *c*, and compare with gel in Fig. 7 *j*) only 10 and 13%, respectively, had stress fibers and these were vestigial (Fig. 8 *c*) compared with control cells (Fig. 8 *b*).

Discussion

Immunofluorescence

Permeabilization of cells followed by immunofluorescence labeling revealed that C4 is continuously distributed along linear actin filament stress fiber bundles (10) in a variety of cells and tissues in all vertebrate species examined so far (human, mouse, rat, chick, frog, and fish). These results were reproduced in cells microinjected with anti-C4 antibody, indicating that the native distribution of these molecules is in association with filamentous actin arrays. Areas of cortical cytoskeleton that are known to contain actin filaments that have a geometrically complex organization (30) were unstained by anti-C4. Similarly, anti-C4 was absent (at the light microscope level) from the mesh work of cross-linked actin filaments that bridge between actin filament bundles more deeply inside the cell (30). Although C4 is absent in skeletal muscle we have recently found that these polypeptides are expressed in myoblasts but are absent when myoblasts differentiate and fuse to form myotubes in culture (Lawson, D., P. Lowings, and C. Shapland, unpublished observations). Interestingly, the epitope recognized by anti-C4 is present in organisms phylogenetically well removed from vertebrates such as molluscs and crustaceans but here the relative molecular masses of the labeled polypeptides are 57 and 18 kD, respectively (unpublished observations). The association of C4 with actin filaments in these species has not yet been determined.

Biochemistry

SDS-PAGE/immunoblotting shows that anti-C4 recognizes a closely spaced doublet (usually found in approximately equal amounts but occasionally with C4^h in excess of C4^l) of M_r 21 kD, the presence of which is unaffected by (*a*) rapid solubilization of cells in SDS, (*b*) high concentrations of reducing agents, and (*c*) a variety of proteolysis inhibitors. The formation of this doublet is thus unlikely to be due to either nonreduced S-H groups or proteolytic degradation. The position of C4 in SDS-PAGE is unchanged in nonreducing conditions suggesting that the C4 polypeptides are monomers or components of noncovalently linked oligomers (21). Detergent extraction of cells with Triton X-100 in a buffer containing 100 mM KCl and 20% ethanediol (34, 36) removes both polypeptides from actin filament bundles, indicating that in these conditions the protein dissociates completely from stress fibers in fibroblasts and is therefore unlikely to be a permanent structural component of actin filament bundles in these cells. However, the association of C4 with actin filaments in fibroblasts could be maintained in many cells, even in the presence of high detergent concentrations by the addition of 20% ethanediol (34, 36), a finding independent of the volume of extraction buffer used. This suggests that the association of C4 with actin filaments is stabilized by both low ionic strength and the presence of ethanediol, as has been found for other actin-binding proteins such as caldesmon, tropomyosin, and calmodulin (34–36, 39, 51). Compelling biochemical evidence for the association of C4 with actin filaments was obtained by immunoblotting standard preparations of smooth muscle actin thin filaments
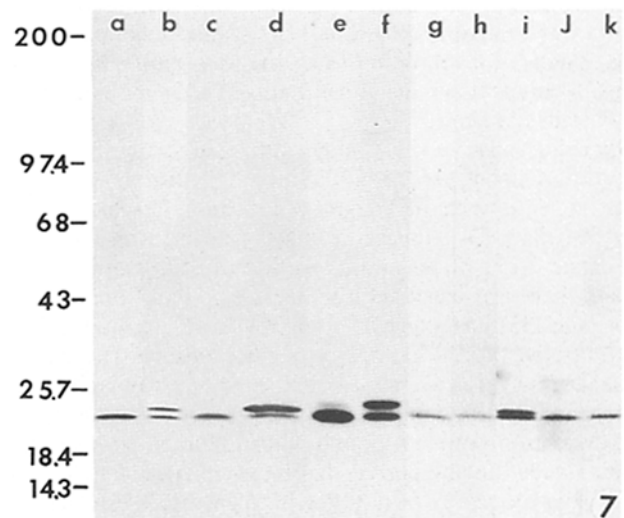
Figure 7. Differential expression of C4 in transformed and shape changed cells. C4^h is absent in SV-40–transformed (*a*) but not normal 3T3 cells (*b*), and RSV ts mutant LA 29 (*c*) but not normal rat 1 fibroblasts (*d*). These virally induced changes in the expression of C4^h are mimicked by suspension culturing 3T3 cells for 72 h after which time only traces of C4^h remain (*e*) whereas both C4^h and C4^l are equally expressed in 3T3 cells cultured normally (*f*). Reexpression of C4^h was not detectable 8 h after cells that had been grown in suspension culture for 72 h (*g*) were allowed to reattach and spread (*h*) but had reached normal levels after 24 h (*i*). This reexpression can be blocked by either actinomycin D (*j*) or cycloheximide (*k*).
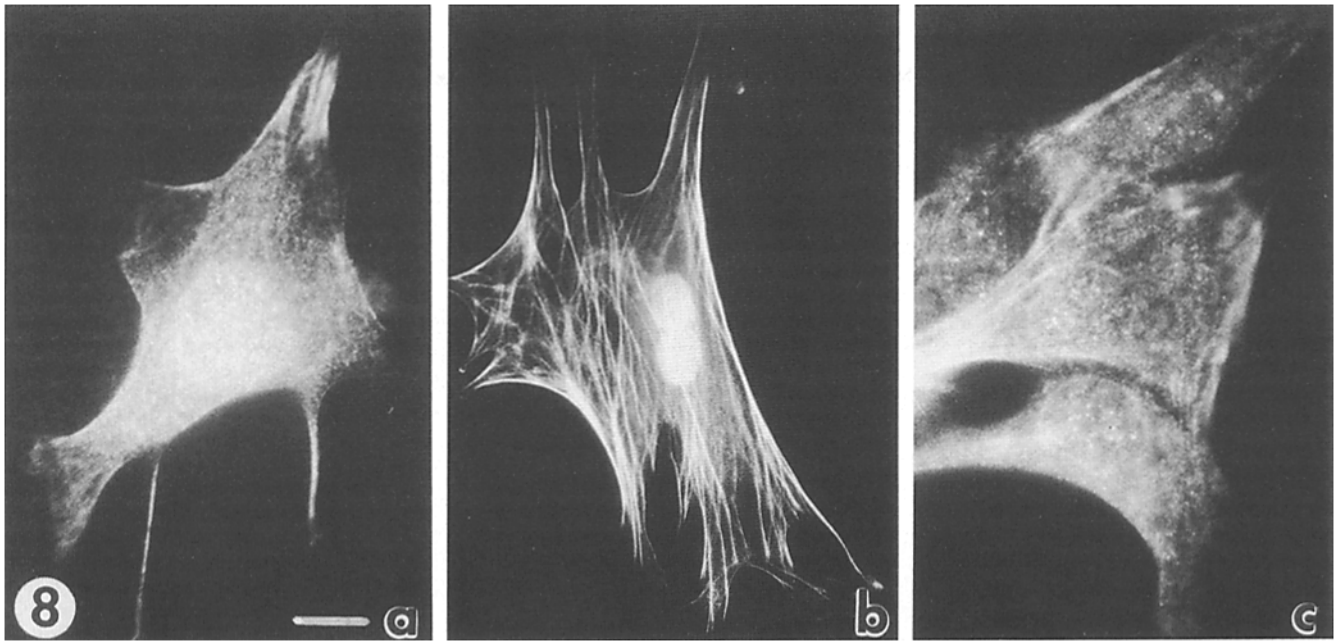
Figure 8. Immunofluorescence of anti-C4 in cells from the same experiment as lanes *g–k* in Fig. 7 show that after 8 h reattachment few large stress fiber bundles were present (Fig. 8 *a* and compare with lane *h* in Fig. 7) many had formed after 24 h in adherent culture (Fig. 8 *b* compare with lane *i* in Fig. 7). However, incubation in either cycloheximide or actinomycin D for the 24-h adherent culture period, which after 72 h in suspension culture, inhibited stress fiber formation in the majority of cells (Fig. 8 *c* and compare with lane *j* in Fig. 7). Bars, 10 μm.

(34) that contain F actin, the actin-binding proteins caldesmon, tropomyosin, and several so far unidentified actin-associated regulatory proteins (37, 46). The removal of these molecules to give F actin filaments parallels the complete loss of anti-C4 labeling and reinforces our observations that the C4 polypeptides are associated, directly or indirectly, with actin filaments.

By using an anti–C4 antibody affinity column we have isolated ~1.5 μg of C4 from 10^6 cells. This (probably lower limit) together with the amount of F actin in fibroblasts (~6 μg/10^6 cells [18]) indicates that the ratio of actin/C4 is of the same order of magnitude to that of actin/tropomyosin where there are seven actin molecules to one tropomyosin molecule (35) and suggests a possibly similar role for C4. As the native protein has not yet been extensively studied we cannot say if C4 is a complex composed of the two different (and noncovalently linked components) or not. We have, however, used in vivo metabolic labeling/immunoprecipitation to study the alternative, that is, whether or not C4^l derives physiologically from C4^h with a precursor/product relationship. Since methionine residues may have been reduced in number or absent in C4^h or C4^l we used [^{3}H]leucine and [^{3}H]lysine and [^{35}S]methionine in immunoprecipitation experiments with no detectable difference in our results. These showed that after a 4-h pulse C4^h had incorporated two to four times as much radioactivity as C4^l. No reduction in this ratio was apparent over a 48-h chase period, suggesting that the longer term turnover/degradation of both epitopes is equal. Furthermore, the lack of any apparent increase in radioactivity present in C4^l and concomitant decrease in C4^h over the 48-h chase period strongly suggests that there is no precursor/product relationship between C4^h and C4^l. SDS-

PAGE immunoblotting showed that C4 does not appear to be related to other actin-associated molecules of similar molecular mass; actin-depolymerizing factor (2), calmodulin (9), chicken gizzard light chain myosin (23), cofilin (40), and 3T3 cell N-ras (53). Furthermore, it is known that N-ras (48, 49) is associated with the cytoplasmic face of the plasma membrane (57). Additional support for our observations is the lack of any homology at the mRNA level between these proteins and C4 (Lowings, P., C. Shapland, and D. Lawson, unpublished observations). In our experiments we have consistently identified a band at 79 kD in heart. However, when heart fibroblasts were dissociated and cultured they did not express this band, indicating that the 79-kD epitope is specific for heart muscle itself. The reasons for this finding and the absence of C4 from skeletal muscle, neurons, and erythrocytes are presently unknown.

Effect of Cell Shape Change and Viral Transformation on C4^h Expression

Cell shape changes whether induced oncogenically or by culture conditions cause marked alterations in actin organization including the loss of stress fiber bundles (1, 3, 7, 12, 43, 54, 55) and a shift in the physical state of actin from F to G (19). Biochemically these cell shape changes are associated with a decrease in the rate of actin synthesis (3, 14). Under these conditions, the α actin isoform gene is known to be switched off completely (in this instance after transformation) leaving at least 500 other of the most abundant polypeptides unaffected by this process (32). Of the 60 or so proteins associated with actin in vertebrate cells (45) immunofluorescence studies have shown that the distribution of myosin,

tropomyosin, α actinin, and vinculin are altered by Rous sarcoma virus and avian sarcoma virus (7, 12, 50). At the biochemical level, vinculin is known to be down regulated fivefold when fibroblasts are cultured in nonadherent conditions (54), several tropomyosins are modified by viral transformation (38), and caldesmon is down regulated by two-thirds (41). However, in no instance is the expression of these molecules switched off completely.

In contrast, we have found that the expression of C4[4] (never C4[1]) is blocked both by cell shape change and transformation (by either DNA or RNA tumor viruses). The fact that 72 h in suspension culture are necessary for the loss of C4[h] is in line with previous studies on the inhibition of protein synthesis in suspension-cultured fibroblasts (5, 6, 14). It has also been shown that suspension culturing cells that are normally adherent blocks them in G1 and that the induction of DNA synthesis requires 14 h after replating and peaks after 20 h (5, 6). In line with these observations, our finding that 24 h after replating is necessary for the reexpression of C4[h] suggests that, unlike actin where synthesis is maximal 8 hrs after replating (14) (indicating the presence of untranslated mRNA [13]), expresssion of the C4[h] epitope is likely to be regulated at the transcriptional rather than the translational or posttranslational level. This was confirmed by incubating cells in either cycloheximide or actinomycin D during the 24-h respreading period after 72 h in suspension culture. These data strongly suggest that the mRNA coding for C4[h], or a molecule associated with this mRNA that affects its stability (8), is, unlike the majority of mRNA species (3, 13), not stabilized against turnover and/or degradation when cells are in suspension culture. Support for this observation comes from our metabolic labeling experiments that showed that after a 72-h chase in suspension culture no labeled C4 was immunoprecipitated (data not shown).

It is known that only a few cell–substratum attachment points and a limited degree of cell spreading are necessary for normal protein synthesis to occur after suspension culture (4). This suggests that the effect of viral transformation or suspension culture on the expression of C4[h] is not simply related to cell shape change since 8 h after reattachment (when cells were well spread) no C4[h] expression was detectable. It is possible, therefore, that the induction of C4[h] expression is associated with some other cellular event. This may well involve the regulation of stress fiber function and/or maintenance of actin stress fiber organization, since these results have shown the concomitant acquisition of C4[h] and stress fiber formation in fibroblasts allowed to respread for 24 h after suspension culture. Furthermore the absence of this polypeptide is associated with (a) the reduction in stress fiber numbers that accompany mesenchymal cell transformation, (b) the acquisition of a rounded morphology and concomitant loss of stress fibers in mesenchymal cells that normally have a full complement of them, and (c) the absence/reduced number of stress fibers in cells such as lymphocytes and epithelial cells. In addition, C4 is absent from areas of the cytoskeleton where actin filaments have a nonlinear, more complex geometric array (30). The function of C4 is still unclear but its presence throughout evolution, modification in different cell and muscle types, and clear-cut alteration during either oncogenesis or cell shape changes strongly suggest that it plays an important but so far undefined cytoskeletal role, the biochemistry and molecular biology of which is currently under investigation.

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