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Biochemistry

Actin microheterogeneity in chick embryo fibroblasts

(isoelectric focusing/smooth muscle/cardiac muscle/myoblast fusion/peptide map)

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ABSTRACT **Secondary chick embryo fibroblasts contain three distinct actin species—α, β, and γ, in the approximate ratio of 1:6:3—with the same molecular weights but different isoelectric points. The most acidic of these components, α, comigrates on isoelectric focusing gels with the major actin of cardiac and skeletal muscle, while γ, the most basic of the actins, comigrates with smooth muscle actin. The three components have overlapping methionine-containing tryptic peptides. All three actins are found to be present in actomyosin and cytoskeleton preparations from chick embryo fibroblasts.**

Identification of α-actin as the major actin from sarcomere-containing cells is confirmed by comparing embryonic chicken pre- and post-fusion myoblast cultures. Following myoblast fusion, the relative amount of α-actin increases until it changes from a minor actin component to the predominant actin species in the culture.

Actin, a major constituent in nonmuscle cells, has been implicated as a participant in a large number of diverse cellular processes. These include cytokinesis (1), which involves actin in the form of a contractile ring around the cell's equator; phagocytosis (1); cell movement on a substratum (1); and possibly the capping of lectin binding sites on the cell surface (2). Cell shape appears to be determined at least in part by a cytoskeletal network of 7-nm-thick microfilaments composed of actin (3). Finally, recent immunochemical and heavy meromyosin decoration experiments have suggested that actin may be a component of the mitotic spindle (4, 5).

Unlike the contractile machinery of striated muscle, in which actin is organized into a stable structure, the actin supramolecular assemblies involved in the events described above are distinctly short-lived. The actin filaments aggregate in specialized ways in response to the constantly changing environmental conditions imposed upon the cell and in response to the demands of the different stages of the cell cycle. One way to explain how actin participates in such a wide range of rapidly changing processes is that within the cell there exist chemically distinct multiple actin species that are differentially utilized in carrying out specific intracellular functions. Here we report evidence of three actin species in chick embryo fibroblasts.

MATERIALS AND METHODS

Materials. Reagents used for isoelectric focusing and sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis were those specified by O'Farrell (6). Trypsin treated with tosylphenylalanine chloromethyl ketone (TPCK-trypsin) was purchased from Worthington, and L-[^{35}S]methionine, 430 Ci/mmol, was obtained from New England Nuclear. Actins from rabbit striated muscle, adult chicken heart, and adult chicken gizzard were prepared by the method of Spudich and Watt (7). All other chemicals were of reagent grade quality.

Cells. Primary cultures of chick embryo fibroblasts (CEF) were the generous gift of Warren Levinson, University of California, San Francisco, and secondary CEF cultures were prepared by S. Brown as previously described (3). Pre- and post-fusion myoblast cultures derived from embryonic chicken breast muscle were obtained from R. Strohman, University of California, Berkeley.

Labeling of Cells. Cells in 60 mm petri dishes were incubated in a 10% CO_2 atmosphere for 3 hr at 37° in a methionine-deficient Dulbecco's modified Eagle's medium plus 10% (vol/vol) horse serum to which was added L-[^{35}S]methionine, 430 Ci/mmol, at 250 μCi per ml of culture fluid. The cells were then washed in phosphate-buffered saline, harvested by scraping and subsequent centrifugation, and immediately dissolved in a lysis buffer containing 9.5 M urea, 2% (vol/vol) Nonidet P-40 detergent, and 0.7 M 2-mercaptoethanol. The washing and harvesting were performed at 4°, and lysed preparations were stored at −70° until ready for use.

Isoelectric Focusing. Isoelectric focusing gels were run according to O'Farrell (6) except that focusing was carried out for 9000 V-hr instead of the usual 6000. The pH gradient was established by 2% (wt/vol) Ampholines of which 80% were pH 5–7 and 20% were pH 3.5–10. After completion of focusing, the gels were fixed in 15% (wt/vol) trichloroacetic acid for 30 min. Ampholines and detergent were removed by shaking in an aqueous solution containing 10% (vol/vol) ethanol and 10% (vol/vol) acetic acid. The gels were then stained with Coomassie brilliant blue, destained by shaking in an aqueous solution of 10% (vol/vol) methanol and 10% (vol/vol) acetic acid, and dried *in vacuo* on Whatman 3MM paper.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. This electrophoresis was carried out in 10% (wt/vol) polyacrylamide slab gels as described by Laemmli (8) and O'Farrell (6). For preparative gels, stained bands were excised from 8 to 12 dried isoelectric focusing gels, pooled, and rehydrated in the O'Farrell $NaDodSO_4$ sample buffer for 30 min. The swollen gel slices were embedded in a layer of melted, $NaDodSO_4$-containing 1% (wt/vol) agarose on top of the slab gel. When the agarose solidified, electrophoresis was performed in the usual manner. Gels were stained with Coomassie brilliant blue but were not then dried.

Tryptic Peptide Maps of [^{35}S]Methionine-Labeled Actin. After purification by isoelectric focusing and $NaDodSO_4$/polyacrylamide gel electrophoresis as described above, the methionine-labeled actin was cut from the $NaDodSO_4$ gel, and stain was removed by shaking with an aqueous solution containing 50% (vol/vol) methanol and 10% (vol/vol) acetic acid. The gel pieces, cut into 1 mm^2 pieces, were then equilibrated with 0.1 M NH_4HCO_3, pH 8.1, and incubated with shaking at 30° for 24 hr with three additions of 18 μg aliquots of TPCK-trypsin. The supernatant solution containing the methionine-labeled tryptic peptides was removed from the gel slices and lyophilized to dryness, and the peptides were redissolved in water. Using 100 μm layers of cellulose, two-dimensional peptide maps were generated by the method of Kempe *et al.* (9) with the following exceptions: 1-butanol/acetic acid/water

Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; CEF, chick embryo fibroblast.

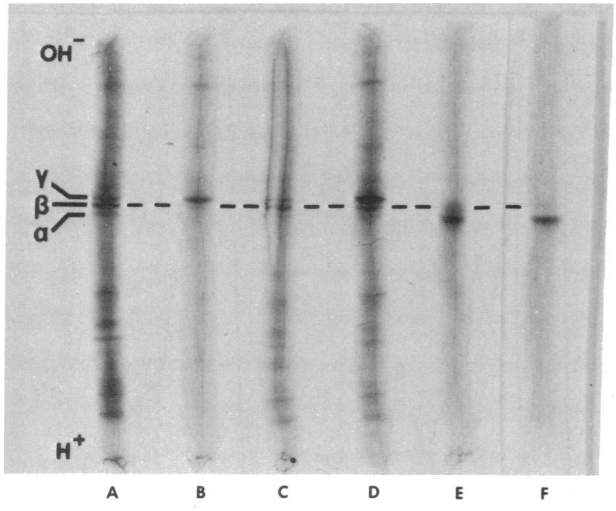

FIG. 1. Isoelectric focusing gels of chick embryo fibroblast whole cell lysate and actin standards from smooth muscle, cardiac muscle, and skeletal muscle, stained with Coomassie brilliant blue. The bottom three-fourths of each gel is shown. (A) Chick embryo fibroblast whole cell lysate, 30 μg of protein. (B) Adult chicken gizzard smooth muscle actin, 2 μg. (C) Chick embryo fibroblast lysate, one-fourth the amount loaded on gel A. (D) Chick embryo fibroblast from gel C plus 1 μg smooth muscle actin from B. (E) Adult chicken cardiac muscle actin, 1 μg. (F) Rabbit skeletal muscle actin, 1 μg.

(4:1:1) was used as the chromatographic solvent, and electrophoresis in pyridine/acetic acid/water, pH 3.5, was carried out for 90 min at 35 V/cm. Radioactive peptides were located by autoradiography.

Cytoskeleton Preparations. Labeled and unlabeled cytoskeleton preparations of CEF cells were made by the method of Brown *et al.* (3).

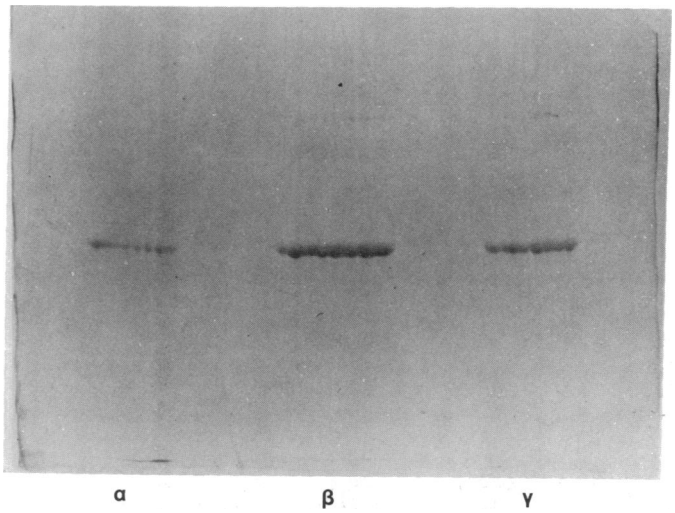

FIG. 2. Preparative $NaDodSO_4$/polyacrylamide gel electrophoresis of the three actin components initially isolated by isoelectric focusing. The gel was run as described in *Materials and Methods* and stained with Coomassie brilliant blue.

Actomyosin Preparation. Actomyosin from CEF cells was prepared through the sucrose step by the method of Clarke and Spudich (10) to give an actomyosin-containing pellet (P2) and an actin-containing supernatant fraction (S2) following centrifugation.

RESULTS

As seen in Fig. 1, isoelectric focusing gels of whole cell lysates of chick embryo fibroblasts revealed three major components in the area of the pH gradient where skeletal muscle actin was known to focus. These proteins were labeled α, β, and γ, in

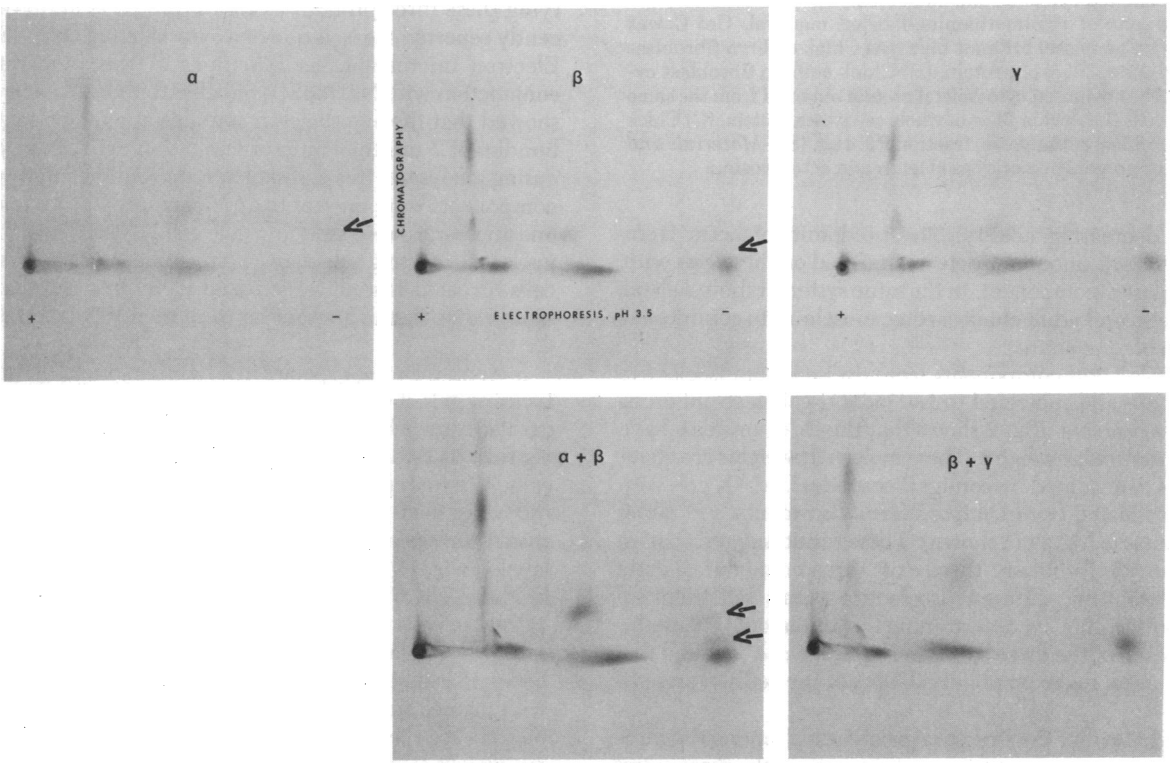

FIG. 3. Tryptic peptide maps of the three [^{35}S]methionine-labeled actin components. Tryptic peptide maps were generated as described in *Materials and Methods*. Maps of mixtures of the components were made by mixing equal amounts of radioactivity of each separate component before application to the cellulose layer. For separate components, 10,000 cpm of protein was applied. For mixtures, 5000 cpm of each component was used.

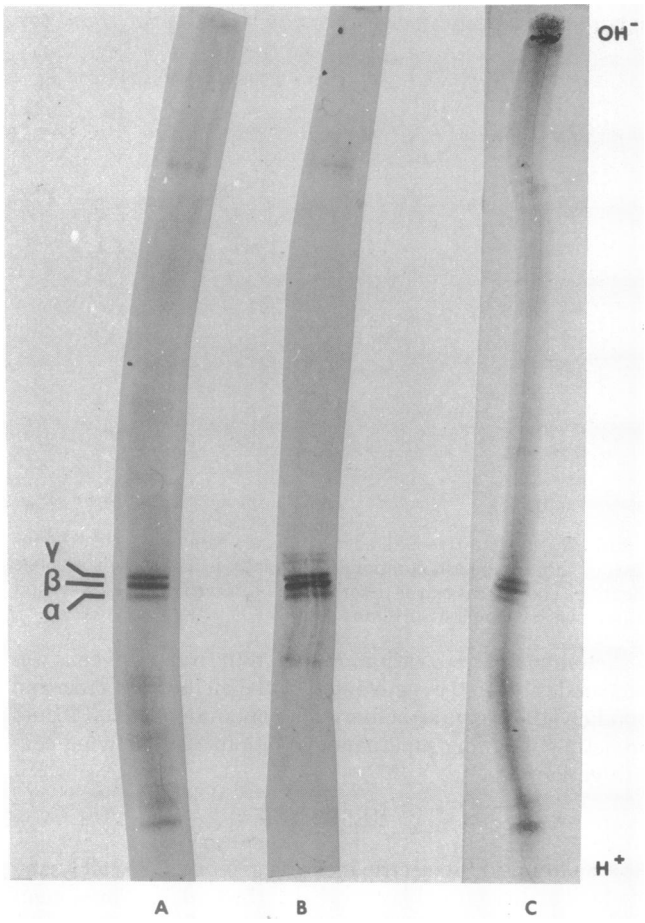

FIG. 4. Isoelectric focusing gels of chick embryo fibroblast whole cell lysate, isolated cytoskeleton, and actomyosin. Gels A and B are autoradiographs of [^{35}S]methionine-labeled material. Gel C was stained with Coomassie brilliant blue. (A) Chick embryo fibroblast whole cell lysate, 30 μg of protein. (B) Chick embryo fibroblast cytoskeleton, the amount of cytoskeletal protein obtained from the same number of cells that yields 40 μg of whole cell lysate protein. (C) Chick embryo fibroblast actomyosin, fraction P2, 4 μg. (See *Materials and Methods* for preparation of cytoskeleton and actomyosin.)

order of decreasing acidity. The predominant actin from chicken gizzard smooth muscle comigrated on these gels with the fibroblast γ-component. In the same system, rabbit skeletal muscle actin and adult chick cardiac muscle actin comigrated with fibroblast α-actin.

The three actin components were excised from isoelectric focusing gels and subjected to $NaDodSO_4$ gel electrophoresis as described above. Fig. 2 shows that the three proteins have identical molecular weights. These molecular weights are about 42,000, as determined by comigration on $NaDodSO_4$ gels with the actins isolated from *Dictyostelium discoideum* and rabbit skeletal muscle (data not shown). These results suggest that, in chick embryo fibroblasts, there exist three nonidentical actin species exhibiting different isoelectric points but identical molecular weights. By determining the amount of [^{35}S]methionine in each of the three bands in Fig. 2, it was also found that β- and γ-actin make up about 80–90% of the actin in these fibroblasts.

To study further the structural relationship among the three components, we made tryptic peptide maps of the three actins isolated from [^{35}S]methionine-labeled cells. By examining the maps of the methionine-containing peptides of the three bands and maps of mixtures of $\alpha+\beta$ and $\beta+\gamma$ (Fig. 3), it is apparent

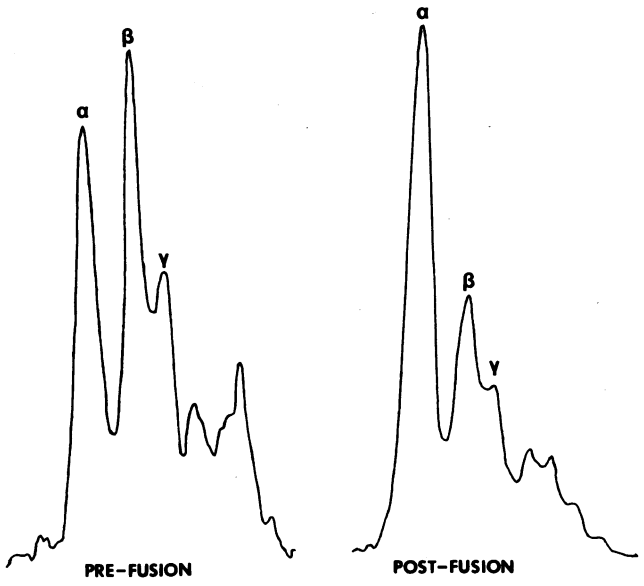

FIG. 5. Scans of isoelectric focusing gels of pre- and post-fusion [^{35}S]methionine-labeled chick embryo myoblasts. Pre-fusion cells were labeled 24 hr after plating. Post-fusion cells were labeled 96 hr after plating as described in *Materials and Methods*. Only the actin-containing portions of the gel scans are shown; the gels were scanned with a GCA McPherson spectrophotometer at 550 nm after staining with Coomassie brilliant blue.

that the maps of the three species are superimposable. A slight difference in the mobility of one peptide in gels of α and β can be seen. The peptide is marked by arrows, and the difference between them is clearly evident in the mixture of $\alpha+\beta$.

We next sought evidence on functional grounds for identifying these three proteins as actins. Brown *et al.* (3) have recently reported the isolation of a cytoskeleton from CEF cells. Electron microscopic examinations of this preparation, in conjunction with $NaDodSO_4$ gel electrophoresis experiments, showed that the cytoskeleton contains as a major component bundles of 7-nm-thick microfilaments of actin. Isoelectric focusing analysis of this cytoskeleton showed that all three actin components were present in approximately the same ratios to one another as were seen in whole cell lysates (Fig. 4). Finally, isoelectric focusing gels of an actomyosin preparation from CEF cells revealed that all three bands were present, again in the same ratios to one another as were seen in whole cells (Fig. 4).

α-Actin is a minor species in CEF cells. Since, on isoelectric focusing gels, it comigrates with actin from skeletal muscle and cardiac muscle but not with smooth muscle actin, we sought to confirm that α-actin is the major actin in sarcomere-containing cells. We therefore examined whether α-actin is synthesized following the fusion of chick embryo myoblasts originally derived from skeletal muscle tissue. Chick embryo myoblasts were labeled with [^{35}S]methionine 24 and 96 hr after plating. At 24 hr, these cells appeared as undifferentiated single cells. At about 44 hr the cells began to fuse, at 72 hr fusion was essentially complete, and at 96 hr the myotubes had grown in size and had begun to exhibit signs of spontaneous contraction. Concomitant with this cell fusion and myotube growth must be the synthesis of actin for the expanding myofibrilar system. Isoelectric focusing gels of lysates of pre- and post-fusion myoblasts (Fig. 5) revealed clearly that following fusion the ratios of the three actins change until α-actin becomes the major component in the culture.

DISCUSSION

Multiple discrete actin species, each participating in its own domain of specific functions, may account partially for the variety of roles played by actin in a nonmuscle cell. Isoelectric focusing of whole cell lysates of CEF cells revealed the presence of three actin species with identical molecular weights but different isoelectric points. The most acidic species, α-actin, accounts for about 10% of the total cell actin, and it comigrates on isoelectric focusing gels with actin from rabbit skeletal muscle and adult chicken heart. In the same gel system, γ-actin, comprising about 30% of the total fibroblast actin, comigrates with the predominant actin species from chicken gizzard smooth muscle.

Two-dimensional tryptic peptide maps of the methionine-containing peptides of all three bands are almost superimposable, indicating the three actin species possess quite similar primary structures. The simultaneous occurrence of identical peptide maps and different isoelectric points for β- and γ-actin is not contradictory. Based on the primary structure of rabbit skeletal muscle actin (11), methionine-containing peptides should account for only about one-fourth of the total peptides produced in a tryptic digest. The amino acids responsible for the different isoelectric points of β- and γ-actin probably reside in the unlabeled peptides. There also may be differences in the unlabeled peptides between α- and β-actin as well.

Studies with [^{35}S]methionine-labeled chick embryo myoblasts before and after fusion showed that in the process of myotube formation the ratios of the three actin components change until α-actin becomes the major actin species in the culture. It is clear, however, that α-actin is not exclusively a sarcomeric actin, because the amounts found in secondary chick embryo fibroblast cultures preclude its being due to myoblast contamination. We must assume, therefore, either that α-actin synthesis in cells that do not contain sarcomeres is the result of a leaky gene control system, or that this actin is required for some special purpose by the CEF cell. We have identified several examples of mammalian non-sarcomere-containing cells that appear to synthesize no α-actin whatsoever (unpublished data).

The simple classification of actin into muscle and nonmuscle types is apparently invalid. Our work shows that the actin of skeletal and cardiac muscle, both sarcomere-containing tissues, is primarily α-actin. Smooth muscle, on the other hand, which does not contain myofibrils but whose chief role is still force generation, contains primarily γ-actin. Furthermore, γ-actin also comprises about 30–50% of the total actin found in so-called nonmuscle cells. We suggest, therefore, that α-actin might be more properly termed *sarcomeric actin* rather than the more commonly used term *muscle actin*.

All three components appear to be involved in biochemical functions associated with actin. Actomyosin isolated from CEF cells contains the three actins in ratios to one another identical to those seen in whole cell lysates. Furthermore, examination of the cell's cytoskeleton, which contains 30–50% of the cell's actin, produced the same results seen with the actomyosin.

In chick embryo fibroblasts, therefore, we have clearly identified three distinct actin species. Two of these three proteins, β-actin and γ-actin, are the dominant species in nonmuscle cells; γ-actin is the predominant component in smooth muscle tissue; and α-actin is the major actin of sarcomere-containing muscle. Our experiments concerning actin function have failed thus far to reveal any gross discrimination among the three species. However, the cell may use multiple actins simultaneously, in the form of hybrid microfilament bundles, to perform a given task. In skeletal muscle, calcium control is exerted at the level of the interactions between troponin, tropomyosin, and actin. On the other hand, in smooth muscle, calcium control is exerted at the level of the myosin itself without the involvement of the troponin system (12). By combining different actins with different control potentials into the same cytoskeletal bundles, the nonmuscle cell may achieve a much wider range of control options to better enable it to respond to the temporal and the environmental demands made on it. Recent work, in agreement with this idea of hybrid bundles, has shown that in nonmuscle cells, tropomyosin (13) and α-actinin (14) bind to bundles of actin microfilaments in a periodic rather than a continuous mode.

While this manuscript was in preparation, we became aware that multiple actin species in nonmuscle cells have recently been independently discovered by Whalen *et al.* (15), Garrels and Gibson (16), and Storti and Rich (17).

The idea that higher cells might contain multiple actin species was first suggested to us by Drs. P. Z. O'Farrell, P. O'Farrell, and R. D. Ivarie on the basis of results they found using a two-dimensional analytical technique to study hormonally and virally induced cell changes. The work in this paper was supported by funds from the Leukemia Society of America (P.A.R.), the American Cancer Society Grant VC121D (J.A.S.) and the Dreyfus Foundation (J.A.S.). P.A.R. is a Special Fellow of the Leukemia Society of America.

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