Selective Isoactin Release From Cultured Embryonic Skeletal Muscle Cells

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ABSTRACT  The culture medium of embryonic quail myoblasts, labeled for 24 h with $[^{35}S]$L-methionine, was analyzed by two-dimensional gel autoradiography. The major polypeptide observed had a 43,000 molecular weight and an isoelectric point of 5.4. This polypeptide could be specifically adsorbed to DNAse-I Sepharose. A tryptic peptide map of the $[^{35}S]$methionine-labeled peptides of intracellular actin and the extracellular major polypeptide were virtually identical. These findings identify the released polypeptide as actin. A comparison of two-dimensional gel patterns of intracellular and extracellular labeled polypeptides showed a large number of differences indicating the actin release did not result from general cellular breakdown. The released actin was not filamentous as judged by its behavior during Bio-Gel A-5m chromatography (Bio-Rad Laboratories, Richmond, Calif.) The released actin did not originate solely from contaminating fibroblasts in the culture because actin was also observed in the medium in clonal myoblast cultures and in purified myotube preparations. Finally, the nonmuscle isoactins, as opposed to muscle $\alpha$-isoactin, were released preferentially. These results indicate that within the developing muscle cell where both muscle and nonmuscle specific isoactins are simultaneously present, the different isoactins may be physically or functionally compartmentalized with the nonmuscle isoactins existing primarily at or near the cell surface.

Materials and Methods

Materials

Tissue culture medium and serum were obtained from Grand Island Biological Co. (Gibco, Grand Island, N. Y.). Chick embryo extract was prepared according to Konigsberg (18). Ampholytes were purchased from LKB Instruments (Rockville, Md.), $[^{35}S]$L-methionine (1,100 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, Ill.) and Enhance was purchased from New England Nuclear (Boston, Mass.) XR-5 x-ray film was bought from Kodak and 100 pL thin layer cellulose chromatography plates from Eastman. TPCK-treated trypsin and DNAse-I Sepharose were obtained from Worthington Biochemical Corp. (Freehold, N. J.). Bio-Gel A-5m and gel backing dialysis tubing were purchased from BioRad Laboratories. All other chemicals were of reagent grade quality.

Fertile eggs of the Japanese quail, Coturnix coturnix japonica, were generously provided by Mr. L. Williams, Department of Poultry Science, Iowa State University, Ames, Iowa.

Muscle Cultures

Breast musculature from 10-d embryonic Japanese quail, Coturnix coturnix japonica, was isolated in Spinner’s saline solution, minced, and mechanically...
dissociated by rapid passage through a Pasteur pipette. The resulting suspension was filtered through boiling silk to give a single-cell population. Cells were washed twice with complete Dulbecco’s modified Eagle’s medium (see below), and plated onto gelatin-coated 35 mm plastic tissue culture dishes at 5 x 10^4 cells/dish.

After 18 h, clonal cultures were established from some of the primary cultures by treating the cells with 0.025% (wt/vol) trypsin for 5 min at 37°C. These released cells were washed, counted, and replated at 200 cells/100 mm tissue culture dish. At the first appearance of myotube formation in the clonal cultures, the cells were washed with Dulbecco’s MEM and selected myogenic clones were encircled by tygon tubing rings that were sealed to the culture dish with silicon grease. The myogenic clone cells were released from the dish by trypsinization as described above, pooled, washed, and replated onto 35-mm culture dishes.

Purified myotubes were made from primary cell cultures by a modification of the procedure of Garrels and Gibson (12, 13). At the earliest stages of myotube formation, cultures were dissociated in calcium-free DMEM containing 0.6% Vickase (Gibco Laboratories) and 1 mg/ml DNAse-I ( Worthington Biochemical Corp.). The DNAse was used by Garrels and Gibson to hydrolyze DNA released when a small percent of the cells were broken during dissociation of the cells. Otherwise, the cells would clump and be no longer fractionated in the next step. No additional cell lysis was observed during the remainder of the procedure. The resulting cells were layered on 30-ml gradients of 10-30% fetal calf serum in DMEM. The cells were allowed to sediment at 1 g for 20 min. The cells at the bottom of the gradient were collected and resedimented through another identical gradient. The recovered cells were predominantly multinucleated and were replated in complete DMEM. To prevent the few remaining myoblasts from replicating, cytosine arabinoside (1.0 μg/ml) was added to the myotube cultures.

All cultures were incubated at 38°C in a humidified atmosphere of 5% CO₂ in air. The growth medium for all cultures consisted of 7% Dulbecco’s MEM, 15% horse serum selected on the basis of supporting clonalgrowth, 10% chick embryo extract, 1% penicillin (10,000 U)-streptomycin (5 mg). When primary cultures were used for labeling experiments, cytosine arabinoside, 1 μg/ml, was added at the end of the second day in culture and removed after the fourth day. Such treatment has been shown to eliminate virtually all replicating mononucleated cells leaving only those cells that had not entered S phase or were postmitotic (9, 28). Microscopic examination of 5-d primary muscle cultures that had been fixed and stained with 0.5% Azure B showed that 75% of the nuclei were in myotubes. Spontaneous contraction of the cells began after day 6.

**Cell Labeling**

After 4-6 d in culture, muscle cells were labeled in 35 mm petri dishes with 0.8 μl of methionine-deficient Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% horse serum and 125 μCi/ml of [35S]methionine (1,100 Ci/mmol) for 6 h at 38°C in an atmosphere containing 5% CO₂. At the end of this time, the labeled medium was removed from the plate and replaced with 0.9 ml of complete DMEM supplemented with 10% horse serum and 2% chick embryo extract. The cells were allowed to continue growing for an additional 17 h. At this time, the medium was removed and centrifuged in a Beckman Microfuge (Beckman Instruments, Inc., Fullerton, Calif.) at 10,000 g for 1 min to remove any cellular debris or floating cells. The remaining cell monolayer was washed twice with cold phosphate buffered saline (PBS). The cells were removed from the plate by gently suspending them in cold PBS with a rubber policeman. They were then washed by centrifugation for 5 min at 2,000 g. The cell pellet was immediately dissolved in 150 μl of a lysis buffer containing 9.2 M urea, 2% nonidet P-40 (NP-40) detergent, 5% mercaptoethanol, and 2% pH 5-7 ampholines as described by O’Farrell (24). 40 μl of the medium were mixed with 170 μl of the same lysis buffer. Samples were stored at -100°C until ready for analysis.

**Gel Electrophoresis**

Two dimensional gel electrophoresis was performed according to O’Farrell (24). The radioactively labeled material was subjected to isoelectric focusing in a pH 5-7 gradient in the first dimension and SDS-gel electrophoresis on 10% polyacrylamide slab gels in the second. One dimension isoelectric focusing (IEF) gels were run in the same manner except that the second dimension was omitted. For the one-dimensional IEF gels, at the end of focusing, the gels were extruded into a 1-M Lactic Acid solution and the pH was adjusted to 3.5 with 1-M sodium hydroxide. The gels were then washed three times with 10% methanol and 10% acetic acid in water over a period of 24 h. The gels were stained with 0.025% Coomassie Brilliant Blue R250 in 10% methanol and 10% acetic acid in water and destained with the same solution. These IEF gels were dried in vacuo between two sheets of dialysis membrane. The two-dimensional slab gels were fixed and stained as described (24).

**Fluorography**

For preparation of fluorograms, the procedure of Chamberlain was used (5). Briefly, the destained gels were washed in a shaking water bath at room temperature for 30 min to remove acetic acid. The gels were then shaken for an additional 45 min in an aqueous solution of 1 M sodium salicylate. The gels were removed, dried in vacuo, and exposed to preflashed Kodak XR-5 x-ray film at -100°C for the desired length of time as described by Laskey and Mills (19).

**Scanning Densitometry**

Fluorograms of gels were quantitated with a Transidyne General RFT-II scanning densitometer containing a peak integrator. Gels were scanned at 550 nm.

**Two Dimensional Peptide Mapping**

To accumulate sufficient intracellular actin for peptide maps, nine two-dimensional gels of [35S]methionine labeled 5-d cultured quail muscle cells were made, and the Coomassie Blue stained actin regions were excised and pooled. For the extracellular actin, 2 ml of medium from labeled cells were passed over a 0.5 ml column of DNAse I-Sepharose as described by Lazarides and Lindberg (20). The actin was eluted with 3 M guanidine hydrochloride and desalted by dialysis against water. After lyophilization, the residue was dissolved in SDS lysis buffer (24) and run on an SDS-polyacrylamide slab gel. The Coomassie Blue stained actin band was then excised.

The gel pieces were minced into 0.5 mm squares and shaken for 8 h in 1 ml of a solution of 0.1 NH₄HCO₃ containing 50 μg of trypsin. The supernatant solution was removed and replaced by an additional aliquot of the same trypsin solution. The suspension was shaken overnight, and the supernatant removed and replaced with 0.5 ml of 0.1 NH₄HCO₃. After an additional 3 h of shaking, the supernatants were pooled and the NH₄HCO₃ removed by lyophilization (25).

**RESULTS**

4- to 6-d-old cultures of embryonic quail breast muscle cells were labeled with [35S]methionine, and the labeled proteins in the medium and in the cells were analyzed by two-dimensional gel electrophoresis. Results of a typical experiment are shown in Fig. 1. The exposure times of the two autoradiograms were adjusted so that the intensity in the actin regions of the gels were equivalent. In the medium, a major polypeptide appears that migrates in the same area of the gel as does actin. Other polypeptides are also released. When compared with the polypeptides seen when the cells are dissolved in 9 M urea, however, the polypeptide pattern is strikingly different. Some of the polypeptides observed in the cell lysate appear in the medium but at reduced levels relative to actin. The arrows depict examples of labeled cellular proteins that are either absent from the medium or present at very reduced levels. Those arrows denoted by the letter S are examples of polypeptides
FIGURE 1 Autoradiograms of intracellular and extracellular \[^{35}S\]\textsuperscript{-}methionine labeled polypeptides. Primary muscle cell cultures were labeled and the samples analyzed by two dimensional gel electrophoresis as described in the text. Shown are fluorograms of the gels. Exposure times were adjusted so that the integrated grain intensities in the actin regions of the gels were equivalent. Plain arrows indicate examples of cellular proteins that are either absent or greatly reduced in the medium relative to actin. Arrows marked by the letter S show samples of polypeptides present in the medium at levels nearly as high as found within the cell. A indicates the position of actin. a) Extracellular polypeptides, 1.6 x 10^8 cpm applied. b) Intracellular polypeptides, 2.4 x 10^8 cpm applied.

seen in the medium at levels approaching those for the polypeptides in the cell.

To further establish that the major peptide observed was not released primarily because of cell lysis, lactic dehydrogenase was assayed in the medium after 24 h and in cells as well (8, 15). By comparing the intra- and extracellular enzyme activity, it could be estimated that at most, ~1-2% of the cells lysed over this time period. On the other hand, as will be shown later, ~14% of the actin synthesized over the same period was released. It seems unlikely that 14% of newly synthesized actin would be released because of lysis of 2% of the cells. Finally monolayers of labeled 5-d primary or clonal myoblast cultures were treated with Triton-X 100 to permeabilize the cell and allow cytosolic and loosely bound membrane proteins to leak into the medium. Such treatments have been shown to leave the cell's cytoskeletal network largely intact (2, 4). The pattern of released proteins here was clearly different than the pattern of proteins released by the cell under normal circumstances (data not shown).

The following properties support the identification of the major released polypeptide(s) as actin. It had a 43,000 mol wt and an isoelectric point in this gel system between 5.2 and 5.4. The polypeptide comigrated on these gels with actin obtained from embryonic chick fibroblast cultures by purification on DNase I-Sepharose. The polypeptide was adsorbed by DNase-I Sepharose, and it could only be eluted with 3 M guanidine hydrochloride (20). Finally, two-dimensional tryptic peptide maps of \[^{35}S\]\textsuperscript{-}methionine labeled intracellular actin and of the medium polypeptide were generated. The results are shown in Fig. 2. The maps of the \[^{35}S\]\textsuperscript{-}methionine labeled peptides from both the intracellular actin and the extracellular polypeptide are almost identical. The observed intensity differences may be a result of the differences in isoactins found inside and outside of the cell as will be discussed.

To determine the molecular state of the released actin, 1 ml of the medium from a labeled cell culture was applied to a Bio Gel A-5m column equilibrated in 50 mM KCl, 1 mM mercaptoethanol, and 50 \(\mu\)M MgATP. Under these conditions, the labeled actin was well included in the column and ran at a position characteristic of G-actin or of short actin oligomers. The results are shown in Fig. 3. As a control, chick skeletal muscle F-actin, 0.5 mg/ml, was chromatographed on the same column and ran well ahead of the released labeled actin. To
further substantiate that the actin observed in the medium was either G-actin or very small actin oligomers, a small sample of medium from labeled cells was centrifuged for 40 min at 160,000 g in a Beckman Airfuge (Beckman Instruments, Inc.). Under these conditions, which control experiments show will clearly pellet F-actin, the released medium actin remained in the supernatant fraction.

Primary cultures of embryonic quail skeletal muscle cells have much lower quantities of contaminating fibroblasts than do corresponding chicken cell cultures (21). Nevertheless, it was important to establish that the released actin was not originating solely with fibroblastic cells. For this purpose, we repeated the experiments with clonal myoblast cultures and purified myotubes. Fluorograms of the medium-containing labeled polypeptides from the clonal cultures and myotubes, shown in Fig. 4, were similar to that observed with the primary cells. These results indicate that the myogenic cells themselves release actin into the medium.

To show that the actin release was not due solely to unfused myoblasts in the culture, 5-d normal cultures and cultures whose fusion had been prevented by inclusion of EGTA in the medium (14) were labeled in parallel with [35S]methionine. The actin released into the medium in each culture was quantitated by scanning densitometry of the two-dimensional gels, and intracellular total protein was determined by TCA precipitable radioactivity in the cells after removal of the medium and washing the monolayer twice with cold PBS. The ratio of released actin to total cell protein was the same in both the fused and unfused cultures (data not shown). This result clearly indicates that actin release is not confined only to nonfused cells in the culture but that the myotubes are equally capable of releasing actin.

Although the major extracellular labeled polypeptide on our two-dimensional gels was clearly actin, the extracellular actin pattern appeared different from that obtained with the intracellular material. In the O'Farrell gel system (24), the second dimension SDS-gel electrophoresis tends to cause a decrease in the isoactin resolution. We thus examined the relative isoactin content of the labeled intracellular and extracellular actins using one-dimensional isoelectric focusing gels in which the α, β, and γ-isoactins are clearly resolved (25).

The presence of large quantities of labeled intracellular desmin, which has an isoelectric point almost the same as actin (16) precluded using urea-solubilized labeled cells directly for the intracellular isoactin analysis. To circumvent this problem, the washed and labeled cells were dissolved in 0.75 M guanidine hydrochloride containing 0.5 mM MgCl2 and 0.5 mM ATP at pH 7 as described by Blikstad et al. (3). Under these conditions, actin is depolymerized but retains its ability to bind to DNase I-Sepharose. The intracellular protein solution obtained by this extraction procedure was applied to a DNase I column, and the actin was eluted with 3 M guanidine hydrochloride (20). This actin, after removal of the salt, and the extracellular actin were analyzed on one-dimension electrofocusing gels. The results are shown in Fig. 5. In the 6-d primary cell cultures (shown here) and the 5-d clonal cultures, there is a much higher proportion of labeled skeletal muscle α-isoactin within the cell than is released compared to the nonmuscle β and γ-isoactins being synthesized at the same time. Therefore, the nonmuscle isoactins are being preferentially released by these cells. These differences were quantitated by scanning densitometry and are shown in Table 1. This release of α-actin is further evidence that the muscle cells and not contaminating fibroblasts are responsible for actin release.

We next determined the percentage of newly synthesized actin that was released over the 24-h period studied. For this purpose, known percentages of the total cellular and extracellular material were run on two dimensional gels, fluorograms were made, and exposure lengths were adjusted so that the degree of film exposure caused by the actin region on each gel was in the linear response range of the x-ray film. The fluorograms were quantitated by scanning densitometry of the actin regions, and the values obtained were corrected for differences in exposure times and in the percent of total intracellular or extracellular material applied to the gel. Final values are presented in Table 2. In the case of 5-d primary cultures, the extracellular labeled actin represents close to 14% of the labeled...
lished results. Westley and Rochefort (27) reported that the myogenic cells. Primary cultures of labeled chick embryo portion of the total labeled actin. Roughly 15% of the labeled actin found in the medium represents a substantial high-purified microvilli (17).

actin as a membrane-actin complex, probably in the form of such membrane fragments present did not pellet a significant proportion of the actin in the medium. Neither did the released actin pellet when centrifuged at 160,000 g in a Beckman Airfuge (Beckman Instruments, Inc.). This agrees with our finding based on gel filtration studies that the extracellular actin is present as G-actin or small actin oligomers and not as F-actin. The fact that actin is present in the medium in its monomeric form does not mean it was released as such from the cell. Serum-containing medium contains actin depolymerizing factors (23), and we have confirmed this for our system as well (data not shown). However, even if serum factors were not present and the actin was deposited initially as F-actin in attachment plaques, its concentration in the medium would be well below the critical concentration required for polymerization, and it would begin to depolymerize.

The most striking finding of our studies is that the nonmuscle specific isoactins are preferentially released by these cells. In prefusion myoblasts, \( \beta \) and \( \gamma \)-nonmuscle isoactins are synthesized (12, 25). However, concomitant with myoblast fusion, synthesis of \( \alpha \)-actin begins and continues until it is the predominant actin in these cells. In mature skeletal muscle, \( \beta \) and \( \gamma \) actins are not found. In our 5- to 6-d old cell cultures, the \( \alpha \)-isoactin accounts for \( \sim 40-50 \% \) of the total labeled actin seen within the cell. In the medium, however, this isomer accounts for \(<20 \% \) of the released labeled actin. In effect, based on our single point determinations, the nonmuscle actins are being released two to three times faster than the muscle-specific isoactin relative to their rates of synthesis. This selective isoactin release is also evidence that the actin release we observe is not the result of general cell breakdown. Detailed kinetic studies are being planned to more accurately determine the time-course of this release.

If all three isoactins were being used equally for the same cellular functions, the relative ratios of the three actin species inside and outside the cell should be identical. If, on the other hand, the \( \alpha \)-isoactin was being specifically recruited for assembly of the developing myofibrils or otherwise compartmentalized, it would be unavailable for equilibration with the general actin pool that is used in carrying out nonmuscle-like cytoskeletal functions. Precedence for selective use of multiple myosin isozymes by a cell has been presented. Gadasi and Korn (11) showed that in Acetabamccoea, two types of myosins were present in different distributions within the cell. Also, in developing myoblast cultures similar to our own, Fallon and Nachmias (7) reported that, on the basis of antibody staining techniques, muscle-specific myosin was found predominantly in the growing myofibrils whereas nonmuscle myosin was selectively used in the cytoskeletal regions near the cell surface. Such differential utilization has not previously been described for the isoactin family. Our results suggest that, in developing myoblasts and myotubes, nonmuscle isoactins are concentrated near the cell periphery.

We have no direct evidence concerning the mechanism of actin release. Cultured nonmuscle cells, while migrating on a substratum, have been shown to deposit actin-containing footpads originating from the attachment plaques by which the cell attaches itself to the petri dish. Developing myoblasts have been shown to contain a cortical layer of microfilaments near the cell periphery (10). If this layer participated in the formation of attachment plaques, cell migration could produce footpads that would then release actin as a result of depolymerization. Our attempts to induce footpad formation in muscle cell cultures by treatment with high concentrations of EDTA (6) were unsuccessful, however. Only 90% of the cells could be released, and the remaining 10% contained so much actin that
footpad analysis was impossible. A second possibility is that actin appears in the medium as a result of exocytosis. Whatever the mechanism, the discrimination described regarding isoactin release suggests strongly that muscle and nonmuscle isoactins may be used differently in developing muscle cells.

We thank Drs. Victor and Rebecca Ionasescu for providing us with embryonic chick muscle cultures used in the initial phases of this study. This research was supported by Muscular Dystrophy Association grants to P. Rubenstein and to A. Sandra and by a grant to P. Rubenstein from the National Institutes of Health (GM-24702). P. Rubenstein is an Established Investigator of the American Heart Association.

Received for publication 13 July 1981, and in revised form 17 August 1981.

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