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 break-down. 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 non-muscle isoactins, as opposed to muscle α-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.

Initial studies by Schubert et al. (26) revealed that a small subset of the proteins made by developing L6 rat myoblast cells were released into the culture medium. This study used one-dimensional SDS-gel electrophoresis to analyze the isotopically labeled proteins released, and collagen was tentatively identified as one of the released polypeptides. This finding was confirmed in additional studies by Lipton (22) and Bailey (1) although none of the additional proteins released were identified. In certain pathological states such as Duchenne dystrophy, muscle has been shown to release, specifically, certain proteins such as creatine kinase, which normally are not seen.

As a prelude to examining polypeptide release by cultured avian dystrophic muscle cells, we wished first to determine which polypeptides are released by normal developing skeletal muscle cells isolated from Japanese quail embryos using high resolution two-dimensional gel electrophoresis. We chose the quail system because primary cultures of these cells have an extremely low fibroblast contamination, and clonal cultures of pure myoblasts can be readily obtained (21). Our results show that actin is one of the major polypeptides specifically released. Furthermore, although muscle and nonmuscle isoactins are being made simultaneously within the cell, the nonmuscle isoactins are preferentially released.

MATERIALS AND METHODS

Materials

Muscle Cultures

Breast musculature from 10-d embryonic Japanese quail, *Coturnix coturnix japonica*, was isolated in Spinner's salts solution, minced, and mechanically

dissociated by rapid passage through a Pasteur pipette. The resulting suspension was filtered through bolting 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 5×10^6 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% Viokase (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% CO_2 in air. The growth medium for all cultures consisted of 74% Dulbecco's MEM, 15% horse serum selected on the basis of supporting clonal growth, 10% chick embryo extract, and 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 cell 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 ml of methionine-deficient Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum and 125 μCi/ml of [³⁵S]L-methionine (1,100 Ci/mmol) for 6 h at 38°C in an atmosphere containing 5% CO_2. 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. The cells were then pelleted 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 15% (wt/vol) trichloroacetic acid and shaken for 30 min. 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 .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 M NH_4HCO_3 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 M NH_4HCO_3. After an additional 3 h of shaking, the supernatants were pooled and the NH_4HCO_3 removed by lyophilization (25). The residue was dissolved in a buffered solution containing pyridine:acetic acid: water (25:1:225), pH 6.5 and applied to a 20 x 20 cm layer of cellulose 100 μm thick. Electrophoresis in the first dimension was carried out in the pH 6.5 pyridine-acetic acid solution just described for 55 min at 400 V. After drying of the solvent, the plates were rotated 90° and developed in a thin layer chromatography tank using n-butanol:pyridine:acetic acid:water (50:33:1:40) as solvent. After drying, the chromtogram of the extracellular material was sprayed with Enhance, a fluorophore, and exposed to preflashed x-ray film at −100°C. The intracellular actin chromatogram was exposed directly to x-ray film at room temperature.

Lactic Dehydrogenase Assay

5-d-old muscle cultures were incubated for 24 h with fresh medium containing serum but no embryo extract. After this time, the medium was removed and centrifuged to remove floating cells and large pieces of cell debris. Medium without cells was treated identically for a control. The cells remaining on the plate were washed in situ with cold Hank's basic salts solution, removed from the dish with a rubber policeman and homogenized in a known volume of buffer. Lactic dehydrogenase activity in the medium and the homogenized cell preparations were assayed spectrophotometrically (8, 15). The low level of activity present in the control medium was subtracted from that of the medium removed from the cells. Degree of cell lysis was calculated by dividing the total units of enzyme activity in the medium by the total activity in the cells taken from the same dish.

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
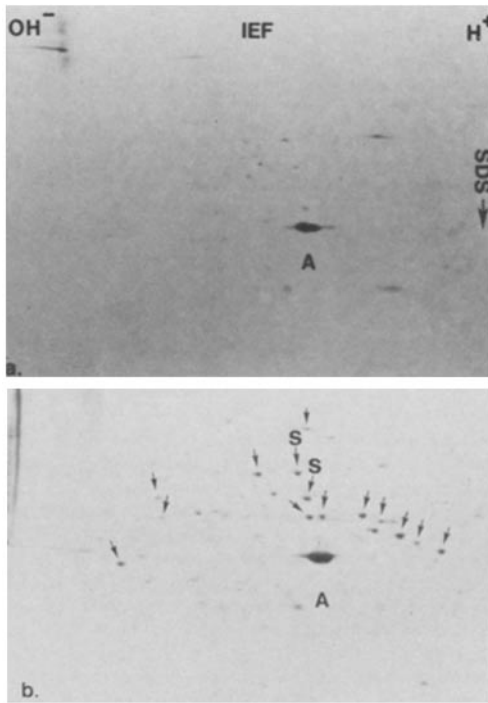
FIGURE 1 Autoradiograms of intracellular and extracellular [35S]-methionine labeled polypeptides. Primary muscle cell cultures were labeled and the samples analyzed by two dimensional gel electrophoresis as described in the text. Shown are fluorograms of the gels. Exposure times were adjusted so that 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 10⁴ cpm applied. b) Intracellular polypeptides, 2.4 X 10⁶ 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 [35S]methionine-labeled intracellular actin and of the medium polypeptide were generated. The results are shown in Fig. 2. The maps of the [35S]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 μM 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
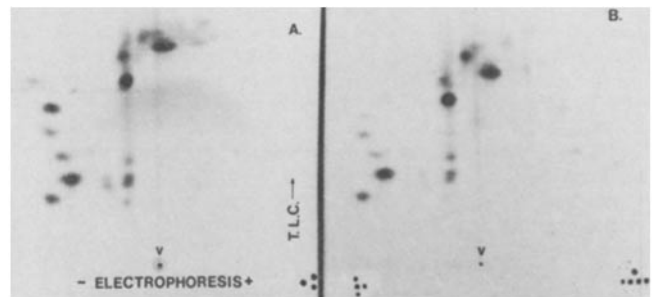

FIGURE 2 Fluorograms of [35S]methionine containing tryptic peptide maps of intracellular and extracellular actin. For experimental details see text. A. Intracellular actin. B. Extracellular actin. The arrowhead indicates the point of sample application for the first dimension electrophoresis.
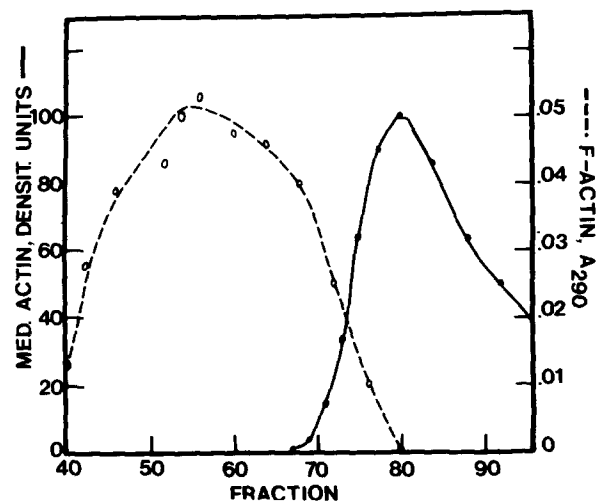

FIGURE 3 Bio-Gel A-5m chromatography of released actin (Bio-Rad Laboratories). To a 1.5 x 57 cm column of Bio-Gel A-5m agarose equilibrated in 0.05 M imidazole·HCl, pH 7.5, containing 1 mM β-mercaptoethanol and 0.2 mM MgATP was applied 0.8 ml of medium taken from 5-d primary muscle cells labeled for 24 h with [35S]-methionine as described in the text. Elution was carried out with the same buffer and 0.5 ml fractions were collected. Fractions were analyzed by SDS-gel electrophoresis and autoradiograms were made. The actin band of each fraction was quantitated by densitometry and intensities were expressed relative to the actin in the peak band (——). As a control, 0.8 ml of a 0.5 mg/ml chick muscle F-actin solution was run on the same column. Actin was monitored by absorbance at 290 nm (- - -).

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 [^{35}S]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 tube 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 $MgCl_2$ 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
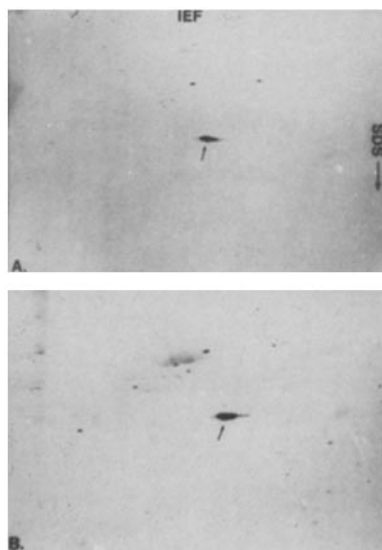

FIGURE 4 Fluorograms of [^{35}S]methionine labeled polypeptides in the medium of clonal myoblast cultures (A) and purified myotubes (B). Cells were labeled as described in the text. ~14 × 10^3 cpm were applied in A and 9 × 10^3 cpm were applied in B.
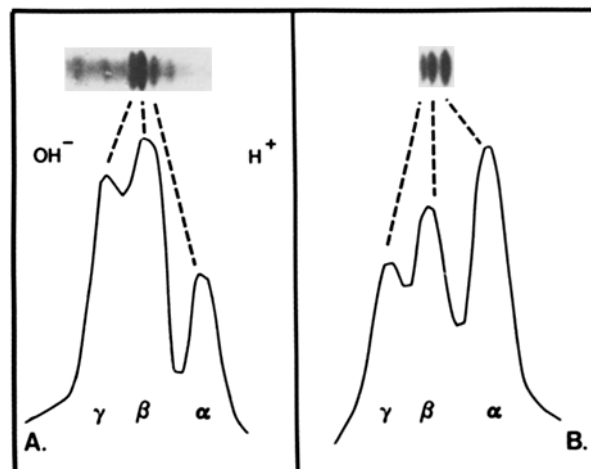

FIGURE 5 Isoactin ratios of the intracellular and extracellular actins from cultured quail primary breast muscle cultures. 6-d quail primary muscle cultures were labeled and samples run on one dimensional isoelectric focusing gels as described in the text. For the intracellular actin, DNAse I-Sepharose chromatography was used to purify the actin before electrofocusing. Autoradiograms of the gels were scanned on a densitometer. The scans and the actin regions of the gels used for the scans are shown. A) Extracellular actin. B) Intracellular actin.

TABLE I

Isoactin Specificity of Released Actin

Sample	$\dfrac{\text{*}\alpha\text{-actin}}{\text{*total actin}}$		$\dfrac{\%\ \text{*}\alpha\text{-actin}_{ext.}}{\%\ \text{*}\alpha\text{-actin}_{int.}}$
	Intracellu-lar	Medium	
6 d primary quail my-oblasts	0.45	0.19	0.42
5 d clonal myoblasts	0.42	0.19	0.45

Cells were labeled with [^{35}S]methionine as described in the text and relative amounts of each of the labeled isoactins was determined by densitometry of the actin bands on autoradiograms also as described.
* [^{35}S]methionine labeling. Total actin is the sum of the α, β, and γ isoactins present in each sample analyzed.

TABLE II

Quantitation of Intracellular and Extracellular
Labeled Actin

Sample	$\dfrac{\text{*actin}_{ext.}}{\text{*actin}_{int.}}$
5-d primary quail myo-blasts	0.14 ± 0.01
5-d clonal myoblasts	0.16 ± 0.01

Cells were labeled with [^{35}S]methionine as described in the text, and the actin regions of two dimensional gel fluorograms of the medium and intracellular labeled proteins were quantitated by scanning densitometry. The results represent the average obtained in two separate experiments.
* [^{35}S]methionine labeling.

actin found within the cell. Considering only the nonmuscle β and γ-isoactins, the labeled extracellular nonmuscle isoactins represent 20% of the labeled intracellular nonmuscle species. In contrast, the labeled extracellular muscle α-isoactin comprises only 7% of the intracellular labeled α-isoactin.

DISCUSSION

Our results clearly demonstrate that actin is one of a number of polypeptides specifically released into the medium by primary cultures of quail embryonic breast muscle cells. Actin release is not due merely to fibroblast cells contaminating the culture because clonal myogenic cultures as well as purified myotubes demonstrate this phenomenon. The amount of labeled actin found in the medium represents a substantial portion of the total labeled actin. Roughly 15% of the labeled actin present in the cells at the end of the 24-h labeling period used in these studies is present in the growth medium.

The phenomenon of actin release, however, is not confined to myogenic cells. Primary cultures of labeled chick embryo fibroblasts exhibit similar behavior (P. Rubenstein, unpublished results). Westley and Rochefort (27) reported that the major labeled polypeptide found in the medium in cultures of MCF$_7$ cells had a 43,000 mol wt and an isoelectric point of ~5.4. Although this polypeptide was not identified, we suspect, based on our results, that it may be actin as well. In another instance, P185 cells could be specifically induced to release actin as a membrane-actin complex, probably in the form of highly purified microvilli (17).

In our study, centrifugation that would probably remove large 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, β and γ-nonmuscle isoactins are synthesized (12, 25). However, concomitant with myoblast fusion, synthesis of α-actin begins and continues until it is the predominant actin in these cells. In mature skeletal muscle, β and γ actins are not found. In our 5- to 6-d old cell cultures, the α-isoactin accounts for ~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 α-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 nonmusclelike cytoskeletal functions. Precedence for selective use of multiple myosin isozymes by a cell has been presented. Gadasi and Korn (11) showed that in Acanthamoeba, 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.

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