Synthesis of polypeptides with the properties of myosin light chains directed by RNA extracted from muscle cultures

(myogenesis/mRNA/wheat germ cell-free system)

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ABSTRACT Polyadenylated RNA, extracted from differentiating primary cultures of rat muscle and the myogenic cell line L8, directs the synthesis of polypeptides in the wheat germ cell-free system which comigrate with myosin light chains under several electrophoretic conditions. The peptides also associate specifically with heavy myosin subunits during dissociation-reassociation treatment. Intact cells of primary skeletal muscle cultures and of the myogenic line synthesize predominantly two light chains. RNA extracted from primary muscle cultures directs the synthesis of a third polypeptide in the cell-free system, similar to the third light chain found in myosin extracted from adult rat thigh muscle. Products of cell-free systems directed by RNA extracted from fibroblasts, reticulocytes, and myeloma cells did not contain detectable amounts of similar polypeptides.

Rat skeletal myoblasts can be grown in cell culture and their transition from proliferation to terminal differentiation can be controlled by the composition of the nutritional medium. The fusion of the cells into multinucleated fibers is followed by a large increase in the rate of synthesis of several muscle proteins and a manyfold increase in activity of enzymes (1, 2).

One of the main problems in investigating the control of these changes (at the molecular level) is the great difficulty in isolating identified mRNA from cells producing so many proteins. A way to by-pass this difficulty would be to elaborate a method for assaying the presence of specific mRNAs in a heterogeneous population of mRNAs. This would provide a relatively simple means to answer at least several questions related to the sequence of events associated with the expression of genetic programs. Earlier studies showed that it is possible to direct, by total cytoplasmic polyadenylated RNA obtained from muscle cell cultures, the synthesis of actin in a wheat germ cell-free system (3). This enabled us to determine the presence of actin messenger in the cells. However, because actin is synthesized in appreciable amounts in many cell types, it is not an ideal marker with which to follow changes during development. Myosin seems more specific to muscle. In skeletal muscle cell cultures, myosin synthesis increases during differentiation, over 20-fold. The active myosin molecule is composed of two large subunits each with a molecular weight of 200,000, and two to three smaller subunits which have molecular weights of from 15,000 to 25,000 (4). The composition of the light chains differs in myosin extracted from different muscle types (i.e., fast, slow, and cardiac muscles), and from muscle of different developmental stages (5).

We wish to report here on the synthesis in the wheat germ cell-free system of polypeptides with electrophoretic mobility and biological properties identical to myosin light chains. The synthesis of these proteins was directed by cytoplasmic polyadenylated RNA extracted from cultures of differentiating primary rat skeletal muscle and by RNA extracted from differentiated cultures of an established myogenic cell line, but not by RNA extracted from fibroblasts, reticulocytes, and myeloma cells.

MATERIALS AND METHODS

Materials. Fresh commercial wheat germ was supplied by the "Bar-Rav" mill, Tel Aviv, Israel and was stored under reduced pressure at 4°.[35S]Methionine (5.6 mCi/ml, 360 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, U.K. Oligo(dT)-cellulose was from Collaborative Research. Nonidet P-40 was a gift from Paz Oil Co., Israel. Rats used were of the Wistar strain.

Cell Cultures of Rat Primary Skeletal Muscle. Cell cultures were prepared and grown as described previously (6). Cultures were grown first in fetal calf serum-embryo extract-enriched medium (PE medium) which promotes cell proliferation without cell fusion. About 40 hr after plating of the cells, the medium was changed to the fusion-permissive S medium. A phase of very intensive cell fusion started about 18 hr after the change to S medium (6).

Myogenic cell line L8 was established in this laboratory from cloned myoblasts isolated during serial passage of primary Wistar rat skeletal muscle cells and grown as described (6).

Fibroblast cultures were prepared from the trunks of rat embryos (15 days or less). The cells were passaged after reaching confluency and tertiary plates were used for extraction of RNA.

All cultures were grown in 100 mm Falcon tissue culture plates.

Preparation of Messenger RNA. Total cytoplasmic polyadenylated RNA was isolated from 15 to 30 cultures by a modification of the method described by Singer and Penman (7). TNM buffer (0.01 M Tris-HCl at pH 7.4, 0.25 M NaCl, 0.01 M MgCl2) was used for rinsing the cultures and TNM buffer containing 1% Nonidet P-40 was used for the lysis of the cells.

Translation of RNA in Cell-Free System from Wheat Germ. Preincubated extracts were prepared, and protein synthesis assays were carried out, as described by Roberts and Paterson (8), except that no tRNA was added and KCl in the reaction mixture was replaced by potassium acetate. [35S] Methionine (25–30 μCi) was added to a 100 μl reaction mixture. Polyadenylated RNA was added to a final concentration of 50 μg/ml. The reaction mixture was incubated for 90 min at 22°.

Isolation of Myosin and Its Subunits. Myosin was purified from the hind legs of adult rats and from muscle cultures (primary and L8), according to Naus et al. (9), except that the high-speed centrifugation to remove the actin was performed in the presence of 10 mM ATP.

Light chains of myosin were prepared by treatment of the

Abbreviations: NaDodSO4, sodium dodecyl sulfate; CPS, cell-free system.
myosin with 2 M urea, as described by Gaffin and Watanabe (10).

Sodium Dodecyl Sulfate (NaDodSO4)/Polyacrylamide Gel Electrophoresis. Cell-free products, preparations of myosin, and myosin light chains were analyzed on NaDodSO4/polyacrylamide gels as described by Laemmli (11), by using a 10–20% polyacrylamide gradient slab gel prepared according to Maizel (12).

Two-Dimensional Gel Electrophoresis. Isoelectric focusing in the first dimension, and NaDodSO4 electrophoresis in the second dimension, were performed according to O'Farrell (13), except that the amphophiles used were of pH range 3.5–10.

Gels were stained and fixed with 50% methanol/7.5% acetic acid (vol/vol) and 0.1% Coomassie brilliant blue, and destained with 5% methanol/7.5% acetic acid (vol/vol).

Measurement of Radioactivity of Specific CFS Products. Dried gels were exposed to an x-ray film for a period of time sufficient to give film blackening proportional to radioactivity. The radioautograph was scanned in a Gilford spectrophotometer at 500 nm, and the intensity of specific peaks was measured by the weight of the paper in the recorded peak.

Dissociation and Reassociation of Myosin Subunits. Dissociation and reassociation of myosin extracted from cultures into its heavy and light chains was done by LiCl treatment, according to Gershman and Dreizen (14) and Low et al. (15).

In the present study, the cell-free reaction mixture was centrifuged at 100,000 × g for 2 hr at 4°C. The supernatant was brought to 0.5 M KCl with solid KCl. Aliquots (200 μl) of the supernatant were mixed with 3 mg fresh myosin from primary muscle culture (the final concentration of myosin was 5 mg/ml, and the final volume of the mixture was 600 μl). The mixture was divided into two parts. One-half was mixed with equivalent volume of 2 mM dithiothreitol in 0.0 M LiCl at 0°C. The second half was mixed with equivalent volume of 2 mM dithiothreitol in H2O at 0°C. The mixtures were immediately subjected to dialysis against KET buffer (0.5 M KCl, 1 mM EDTA, 10 mM Tris at pH 7.4) as described by Low et al. (15). Myosin was then isolated from the mixtures by two precipitations of the myosin in 0.05 M KCl. The myosin was analyzed on NaDodSO4/polyacrylamide gel. The gels were radioautographed, and the amount of light chains was calculated from the scan.

RESULTS

Light chains of myosin extracted from muscle cultures

Myosin extracted from leg muscle of adult rat contains three very distinct light chains. The molecular weight of these chains, calculated from their mobility on 12.5% acrylamide/NaDodSO4 gels, is 23,000, 17,000, and 15,000. Myosin extracted from primary rat skeletal muscle cultures and from differentiated L8 cultures contains the two heavier light chains; the third light chain is not detectable or present in much smaller amounts (Fig. 1). The failure to detect the third chain in myosin extracted from rat muscle culture has been reported previously (16).

Cell-free protein synthesis directed by polyadenylated RNA extracted from cultures

Total cytoplasmic polyadenylated RNA extracted from muscle culture stimulated a 25-fold increase in the incorpora-
tion of [35S]methionine into the trichloroacetic acid-insoluble material synthesized in the wheat germ cell-free system (CFS).

Analysis of the products of the CFS on NaDodSO4/polyacrylamide gels is shown in Fig. 2. The peptides range in size from 10,000 to 100,000 daltons (heavier peptides are not produced in this CFS).

The products formed by CFS to which mRNA from the myogenic line L8 culture was added contain two bands which comigrate with two myosin light chain markers of 23,000 and 17,000 molecular weight. The two bands are designated here by LC1 and LC2, respectively (Fig. 2). These two bands are very distinct among products of RNA extracted from differentiated L8 culture when compared to products of RNA extracted from L8 culture harvested at the early stages of differentiation (proliferating cells). These two bands comigrate with the light chain markers both in two-dimensional gel (isoelectric focusing followed by NaDodSO4 gel electrophoresis) (Fig. 3) and in 8 M urea gel at pH 8.7 (data not shown). A distinct band which migrates close to the LC1 band is present also in products formed by RNA extracted from fibroblast cultures. However, this band is a little heavier (Fig. 2) and it does not migrate as LC1 in urea gel.

Radioactive peptides comigrating with LC1 and LC2 in all three electrophoretic conditions were also found among the products of CFS directed by RNA from differentiated primary muscle cultures, although their proportional amount is smaller than among CFS products directed by RNA extracted from differentiated L8 cultures. In addition, products of CFS directed by this RNA contain a relatively large amount of a third polypeptide which comigrates with LC3 (Figs. 2 and 3). Products of CFS directed by RNA extracted from primary muscle cultures grown in FE medium (consisting only of proliferating mononucleated cells) did not contain detectable amounts of peptides comigrating with LC1 and LC2 in two-dimensional (isoelectric focusing-NaDodSO4) gel and urea gel. However, a band comigrating with LC3 was detectable in all three electrophoretic conditions.

Ability of CFS products to associate with myosin carrier

The association of polypeptides with myosin during treatment which causes dissociation and reassociation of myosin subunits serves as a means of identifying myosin light chains and testing their activity (15, 17). To test the ability of the CFS products to associate with myosin heavy chain, we mixed radioactive products of CFS with unlabeled myosin extracted from primary cultures, and subjected them to conditions which favor dissociation and then reassociation of the myosin subunits, as described in Materials and Methods. Myosin was then extracted, purified, and assayed on NaDodSO4 gels. Radioautography of the gels was performed. The radioautograph of such myosin contained very few bands. Three of these bands comigrated with the light chain markers, and one with actin. The light chain bands were much darker in the radioautograph of myosin subjected to dissociation and reassociation treatment whereas the other bands were unaffected (Fig. 4). Densitometric measurement of the intensity of the bands showed that myosin dissociated and reassociated in the presence of CFS products, directed by mRNA from differentiated cultures, contained eight to nine times more radioactive light chains than myosin added to the CFS products and purified without dissociation-reassociation treatment (Table 1). No radioactive light chains were found in myosin dissociated and reassociated in the presence of products of CFS directed by RNA obtained from myeloma cells, reticulocytes, or fibroblasts (Fig. 4c and c').

DISCUSSION

The data show that polyadenylated RNA extracted from differentiating muscle cell cultures directs the synthesis of peptides which have electrophoretic mobilities, in NaDodSO4/acylamide gels, identical to the light chains of myosin extracted from such cultures. These peptides also comigrate with the light chains in 8 M urea gels and in two-dimensional gel electrophoresis (isoelectric focusing and NaDodSO4). No detectable amounts of such peptides were found in the products of CFS directed by RNA extracted from fibroblasts, reticulocytes, or myeloma cells. CFS products which comigrate with the light chain markers become associated specifically with the myosin carrier during treatment which causes dissociation and reassociation of the light and heavy myosin subunits. It is therefore strongly suggested that, in the CFS, RNA directs the synthesis of the complete, functional light subunits of myosin.

* Preparations of RNA from L8 and primary muscle cultures were also translated in mouse Krebs II ascites CFS (20). The products migrated on NaDodSO4 gels in a pattern very similar to the wheat germ CFS products. Very distinct bands migrated exactly as the light chain peptides produced in the wheat germ CFS.
Table 1. Effect of dissociation and reassociation of myosin subunits on the association of labeled CFS products with myosin

<table>
<thead>
<tr>
<th>RNA from primary muscle cultures</th>
<th>Treated (A)*</th>
<th>Untreated (B)</th>
<th>A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>1.7</td>
<td>1.7</td>
<td>1</td>
</tr>
<tr>
<td>LC1</td>
<td>11.1</td>
<td>1.3</td>
<td>8.5</td>
</tr>
<tr>
<td>LC2</td>
<td>12.5</td>
<td>1.4</td>
<td>8.9</td>
</tr>
<tr>
<td>LC3</td>
<td>8.9</td>
<td>1</td>
<td>8.9</td>
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</tbody>
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For legend, see Fig. 4.
* Subjected to dissociation and reassociation treatment.

Myosin from chick embryo skeletal muscle contains predominantly two light chains. The third peptide is either missing or present in small amounts, and it increases in amount only several days after hatching (18, 19). Similar results were reported for rabbit myosin (5). A peculiar observation is the discrepancy between the relative synthesis of LC3 in the intact cells and the synthesis of a similar polypeptide in the CFS. Myosin extracted from L8 cells does not contain detectable amounts of LC3. In primary skeletal muscle cultures, the synthesis of this protein becomes detectable only in well-differentiated cultures and even then the rate of its synthesis is less than one-sixth of the other two light chains (unpublished). Yet, RNA extracted from primary cultures—including early stages of differentiation—did direct, in the CFS, the synthesis of relatively large amounts of a radioactive peptide which comigrates with LC3. This polypeptide associates specifically with myosin during dissociation and reassociation treatment. The fibroblasts which contaminate the primary muscle cultures contain small amounts of myosin. However, no peptide which comigrates with the third light chain of the muscle myosin could be found in this myosin (unpublished). Also, RNA from these fibroblasts did not direct the synthesis of LC3 peptide in the CFS. These data, although preliminary, may suggest that either the messenger for the third light chain is present in the intact cell in a relatively unexpressed state and the regulation of the amount of synthesis is made on a post-transcriptional level, or the mRNA for the third light chain is translated in the CFS with much higher efficiency than the mRNA for the other two chains. Another possibility is that this protein is synthesized in the cells but does not become associated with myosin heavy chains, and is therefore lost during the process of purification.

The ability to assay the presence of mRNAs for the myosin subunits in a CFS makes possible the study of questions regarding the control of synthesis of these proteins during differentiation.


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