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Biosynthesis of Intrinsic Sarcoplasmic Reticulum Proteins during Differentiation of the Myogenic Cell Line L6*

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Temporal patterns of biosynthesis and the rates of turnover of the 53,000- and 160,000-Da glycoproteins from the sarcoplasmic reticulum membrane were determined and compared with the rates of biosynthesis of the (Ca^{2+} + Mg^{2+})-dependent ATPase in the differentiating rat myogenic continuous cell line, L6. Cells were labeled at various stages of differentiation with [^{35}S]methionine and sarcoplasmic reticulum proteins were isolated from Triton extracts of the cells by protein A-mediated immunoprecipitation with specific antibodies. The immunoprecipitates were separated by polyacrylamide gel electrophoresis and the radioactivity incorporated into particular protein bands was recorded by autoradiography.

The patterns of biosynthesis and the rates of degradation of the three intrinsic sarcoplasmic reticulum proteins were similar, even though two of them were glycosylated and one was not.

The glycoproteins and the ATPase were found in the microsomal fraction obtained from the cell homogenate but not in the cytosol. This suggests that the synthesis and processing of these intrinsic sarcoplasmic reticulum proteins occurred in membrane systems.

Sarcoplasmic reticulum, the membrane which regulates the concentration of calcium ions in muscle fibers during the process of contraction and relaxation (1, 2), contains several well characterized proteins (3). The intrinsic sarcoplasmic reticulum proteins include the (Ca^{2+} + Mg^{2+})-dependent ATPase (4), a proteolipid (3), and two glycoproteins with molecular weights of 53,000 and 160,000 (5). The sarcoplasmic reticulum also contains two extrinsic proteins, calsequestrin (6) and the high affinity calcium binding protein (7).

The biosynthesis of these different sarcoplasmic reticulum proteins and their assembly into membranes in differentiating muscle has been of interest to us for several years. We have compared the process of biosynthesis of the (Ca^{2+} + Mg^{2+})-dependent ATPase, calsequestrin, and the high affinity calcium binding protein in differentiating primary cultures of rat skeletal muscle cells (8-10). We found that biosynthesis of the ATPase was turned on at about the same time that cell fusion began (8, 9). Its synthesis was not coordinated with the biosynthesis of calsequestrin (9, 10) or the high affinity calcium binding protein (10), which were turned on about 20 h earlier. The pattern of biosynthesis of the two extrinsic proteins was, however, almost identical, even though calsequestrin is glycosylated and the high affinity calcium binding protein is not. These biochemical findings were in agreement with our morphological studies using indirect immunofluorescence as a test for the appearance of the protein in cultured cells (11). Consequently, it was of interest to us to compare the patterns of synthesis of the glycosylated and unglycosylated intrinsic proteins of the sarcoplasmic reticulum membrane. This paper presents data showing that biosynthesis of the intrinsic glycoproteins and the (Ca^{2+} + Mg^{2+})-dependent ATPase occurs in a coordinated manner during differentiation of L6.

MATERIALS AND METHODS

Maintenance and Labeling of Cell Cultures—The continuous myogenic cell line, L6, originally isolated by Yaffe (12) from primary cultures of rat skeletal muscle, was used in this study. Cells were plated at a density of 10^6 cells/tissue culture flask (75 cm^2) and grown as described previously (13) in 25 ml of Dulbecco's modified Eagle's medium supplemented with 10% horse serum and containing 10 μg of gentamycin/ml. Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was routinely changed on the second day after plating and every third day thereafter.

Cells were labeled for 4 h at various stages of differentiation with [^{35}S]methionine (specific activity 1000 to 1300 Ci/mmol) added at a concentration of 10 or 20 μCi/ml. The labeling medium contained cold methionine at a concentration of 4 μM.

Degradation rates were obtained as described previously (8) using cells collected at different time intervals after a 24-h labeling period with [^{35}S]methionine.

Preparation of Antisera—Preparation of the antisera against the 53,000-Da glycoprotein (14) and the (Ca^{2+} + Mg^{2+})-dependent ATPase (8) has been described previously.

Isolation of Intrinsic Sarcoplasmic Reticulum Proteins—Labeled cells were washed three times with 10 ml of ice-cold 0.015 M citrate, pH 7.8, 0.15 M KCl and harvested by scraping with a rubber policeman. The cell pellet, collected by centrifugation at 800 × *g* for 4 min, was dissolved in a solution of 0.5% or 1% Triton X-100, 50 mM Tris-HCl,

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pH 7.5, 150 mM NaCl, and 100 KIU[1] of Trasylol/ml and centrifuged for 1 h at 100,000 × *g*.

Sarcoplasmic reticulum proteins were isolated from the Triton extracts of cells by protein A-mediated immunoprecipitation with specific antibodies. Samples of cell extracts containing 200 μg or 250 μg of cellular protein were mixed with a solution of 1% Triton, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.2, and 100 KIU of Trasylol in a total volume of 1 ml. Immunoprecipitates were obtained by incubation of this mixture with 10 or 20 μl of antiserum and 350 μl of a 10% suspension (w/v) of protein A-Sepharose beads in a solution containing 1% Triton, 150 mM NaCl, 50 mM Tris-HCl, pH 7.2, and 100 KIU/ml of Trasylol as described earlier (14, 15). Sepharose beads with bound immunoprecipitates were collected by centrifugation and washed five times with 1 ml of a solution of 100 mM Tris-HCl, pH 7.5, 200 mM LiCl, and, in some cases, 0.2% 2-mercaptoethanol and twice with 1 ml of a solution containing 1 mM Tris-HCl, pH 7.5, and 150 mM NaCl.

A second immunoprecipitate was obtained from the same extract by further incubation with a new portion of beads and a new portion of the appropriate antiserum. Immunoprecipitates were extracted from the protein A-Sepharose beads by boiling for 2 min in 100 μl of SDS electrophoretic sample buffer and used for SDS-gel electrophoresis. SDS-polyacrylamide electrophoresis in 8.5% Laemmli slab gels (16) was carried out for 16 h with a constant voltage of 80 mV. Sarcoplasmic reticulum proteins used as standards were run in parallel with the immunoprecipitates in the same gel. Proteins were stained with Coomassie blue, destained, and, after drying, gels were exposed for autoradiography at −20 °C using Kodak SB-5 x-ray films.

In some experiments quantitation of radioactivity in the protein bands was achieved by cutting out radioactive bands, dissolving them in 300 μl of 30% H_2O_2 by heating at 50 °C overnight and, after the addition of 10 ml of scintillation fluid, counting them in a scintillation spectrometer. Background counts were established by counting gel sections of the same lanes. Background values were subtracted from the radioactivity in the protein bands and the results were expressed as counts per min/mg of the total protein in the Triton extracts of the cells.

Removal of Carbohydrate Chains with Endo H—Immunoprecipitates were extracted from Sepharose beads by boiling the packed pellet of beads for 2 min with 5 μl of 10% SDS. After cooling, the SDS concentration was reduced by dilution with 200 μl of 50 mM sodium citrate, pH 5.5. Samples containing 1 mM phenylmethylesulfonyl fluoride were incubated for 16 h at 37 °C with 10 milliunits of Endo H according to the method described previously (14). Samples of immunoprecipitates without Endo H were incubated in parallel. After incubation, 80 μl of SDS sample buffer (60% glycerol, 30% 2-mercaptoethanol, 6% SDS, 0.2 M Tris-HCl, pH 6.8, and bromphenol blue) were added to the mixtures and the proteins were separated by gel electrophoresis in the Laemmli system (16).

Radioiodination of Sarcoplasmic Reticulum Proteins—Sarcoplasmic reticulum proteins were radioiodinated as described by Habener *et al.* (17) using Bio-Rad Enzymobeads with covalently attached lactoperoxidase and glucose oxidase.

Radioiodination of Protein A—Protein A was radioiodinated according to Greenwood *et al.* (18) with some modifications. Iodination was carried out at room temperature in 50 μl of a solution containing 100 μg of protein A, 150 mM NaCl, 10 mM sodium phosphate, pH 7.2, 10% dimethyl sulfoxide, 1 mCi of $Na^{125}I$, and 0.05% chloramine-T. The reaction was stopped after 15 min by the addition of 5 μl of 0.1% sodium metabisulfide solution. Radioiodinated protein A was purified by chromatography on two Sephadex G-25 columns. The first column was equilibrated with 150 mM NaCl and 10 mM sodium phosphate, pH 7.2, the second with 50 mM sodium phosphate, pH 4.0. Radioiodinated protein A was stored at −20 °C in a solution of 50 mM sodium phosphate, pH 4.0, and 40% ethanol.

Electrophoretic Blotting Procedure and Immunological Detection of Proteins on Blot—Sarcoplasmic reticulum proteins were subjected to electrophoresis in 8.5% SDS-polyacrylamide gels (16). Proteins were then transferred electrophoretically from the gel to nitrocellulose sheets according to Towbin *et al.* (19). The electrophoretic blots were incubated for 1 h at 37 °C with 3% bovine serum albumin in saline buffer (150 mM NaCl and 10 mM Tris-HCl, pH 7.2) and washed 5 times with saline buffer at room temperature. The blots were incubated in 3% bovine serum albumin in saline buffer for 5 h at room temperature with a 1:100 dilution of the antiserum prepared against the 53,000-Da glycoprotein. The blots were then washed with saline buffer and incubated for 16 h with radioiodinated protein A (10^6 cpm/ml). Finally, the blots were washed with saline buffer, dried, and exposed for 24 h to Kodak X-Omat R film at −70 °C.

Measurement of the Rate of Total Protein Synthesis—Incorporation of [^{35}S]methionine into total protein was measured by trichloroacetic acid precipitation of Triton extracts of cells. The precipitate was recovered by a filter paper disc method (20). The results were expressed as counts per min/mg of total cellular protein.

Fractionation of Homogenates of L6—L6 myotubes (8 days after plating) were labeled for 4 h with [^{35}S]methionine and then isolated and homogenized with 15 to 20 strokes in a Dounce homogenizer in 5 volumes of a solution containing 0.3 M sucrose, 50 mM Tris-HCl, pH 7.5, and 100 KIU of Trasylol/ml. The efficiency of cell breakage was observed in a phase contract microscope. The cell homogenate was centrifuged sequentially for 15 min at 10,000 × *g* and for 1 h at 100,000 × *g*. The "microsomal" pellet, precipitated between 10,000 and 100,000 × *g*, was resuspended in the buffer used for homogenization. The 100,000 × *g* supernatant was dialyzed overnight against 5 mM Tris-HCl, pH 7.5, and 0.1 mM phenylmethylesulfonyl fluoride and then was recentrifuged and concentrated by lyophilization. All steps of the fractionation procedure were carried out at 4 °C.

Before immunoprecipitation, samples of the microsomal fraction containing 250 μg of protein were dissolved in 1 ml of a buffer containing 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.2, and 100 KIU of Trasylol. The same buffer was added to aliquots of the 100,000 × *g* supernatant containing 250 μg of protein.

Preparation of Sarcoplasmic Reticulum Vesicles—Sarcoplasmic reticulum vesicles were prepared from rat or rabbit skeletal muscle according to the method of MacLennan (4) as modified by Campbell and MacLennan (5).

Preparation of (Ca^{2+} + Mg^{2+})-dependent ATPase—The (Ca^{2+} + Mg^{2+})-dependent ATPase was isolated from rat sarcoplasmic reticulum according to the method of MacLennan (4) except that the ammonium acetate fractionation was carried out at pH 8.35. Protein was determined by the method of Lowry *et al.* (21).

RESULTS

Characterization of the Antiserum and Identification of the Immunoprecipitated Proteins—Fig. 1*A* shows the protein

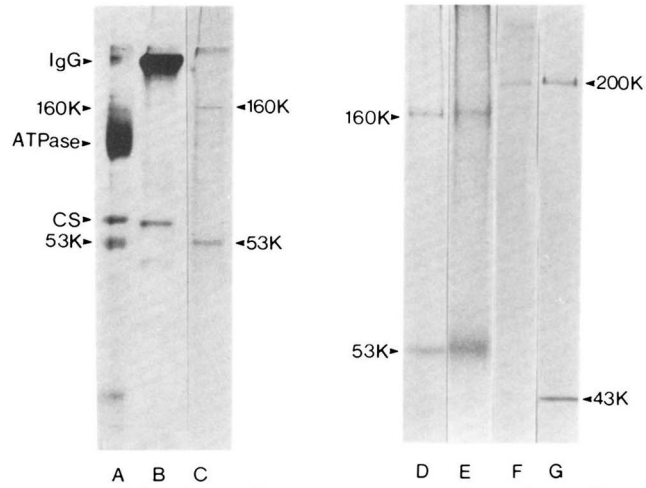

FIG. 1. **SDS-polyacrylamide gel electrophoretic profiles of immunoprecipitates isolated from cultures of L6 with an antiserum prepared against the 53,000-Da glycoprotein.** Samples were separated in 8.5 and 7.5% Laemmli SDS-polyacrylamide slab gels in the absence of 2-mercaptoethanol. *A*, rat sarcoplasmic reticulum proteins stained with Coomassie blue. *B*, immunoprecipitate isolated with protein A beads after incubation of a [^{35}S]methionine-labeled extract of L6 myotubes with 20 μl of antiserum against the 53,000-Da glycoprotein. The gel was stained with Coomassie blue. *C*, autoradiogram of *gel B*. *D*, immunoprecipitate formed between radioiodinated rabbit sarcoplasmic reticulum proteins and antiserum against the 53,000-Da glycoprotein. *E*, similar to lane *C*. *F*, autoradiogram of the second immunoprecipitate obtained from the cellular extract in *D* with an additional 20 μl of antiserum. *G*, autoradiogram of the immunoprecipitate obtained from a [^{35}S]methionine-labeled cellular extract with 10 μl of preimmune sheep serum.

[1] The abbreviations used are KIU, Kallikrein inactivating units; SDS, sodium dodecyl sulfate; Endo H, endo-β-*N*-acetylglucosaminidase H.

pattern of the rat muscle sarcoplasmic reticulum that was used as a standard for identification of sarcoplasmic reticulum proteins immunoprecipitated with antibodies. Fig. 1*B* shows an immunoprecipitate obtained with the antibody prepared against the 53,000-Da glycoprotein of sarcoplasmic reticulum. The molecular weight of this glycoprotein was very close to the molecular weight of the heavy chain of immunoglobulin. In order to avoid an overlap of these two protein bands in the gel, which made identification of the sarcoplasmic reticulum protein difficult, gel electrophoresis was routinely performed in the absence of reducing agents. Fig. 1*C* is an autoradiogram which shows that the 53,000-Da protein and 160,000-Da protein in the immunoprecipitate and the protein standards (Fig. 1*A*) ran in an identical position when gel electrophoresis was performed in the absence of reducing agents.

Fig. 1*D* shows that the antiserum raised in sheep against the 53,000-Da glycoprotein from rabbit sarcoplasmic reticulum reacted with two proteins present in rabbit muscle sarcoplasmic reticulum vesicles, the 53,000 and 160,000-Da glycoproteins. The same molecular weight proteins were observed when gel electrophoresis was carried out in the presence of reducing agents. Although the antiserum was prepared in sheep against the rabbit glycoprotein, it cross-reacted with rat proteins. As shown in Fig. 1, *C* and *E*, the antiserum caused precipitation of both the 53,000- and the 160,000-Da glycoproteins from the Triton extract of [^{35}S]methionine-labeled L6 myotubes. The antiserum did not react with the 105,000-Da (Ca^{2+} + Mg^{2+})-dependent ATPase nor with the 63,000-Da calsequestrin (Fig. 1, *C*, *D* and *E*). Fig. 1*F* shows that only negligible radioactivity was present in the gel of the second immunoprecipitate, indicating that quantitative recovery of the newly synthesized proteins was obtained in the first immunoprecipitate. The 53,000- and 160,000-Da proteins from the cellular extract were not precipitated with the preimmune serum (Fig. 1*G*). Two proteins that did bind nonspecifically to the beads after incubation of the Triton extract of cells with the preimmune serum probably corresponded to myosin and actin, based on their molecular weights (Fig. 1*G*).

Fig. 2 is an autoradiogram of a nitrocellulose blot of rabbit

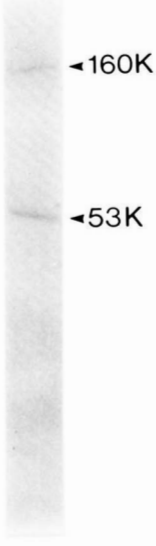

FIG. 2. **Immunoreactivity of sarcoplasmic reticulum glycoproteins with the antiserum prepared to the 53,000-Da glycoprotein after transfer to a nitrocellulose sheet.** Sarcoplasmic reticulum proteins, separated in an SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose, were incubated with an antiserum prepared against the 53,000-Da glycoprotein and then with radioiodinated protein A as described under "Materials and Methods." An autoradiogram of the nitrocellulose sheet is presented.

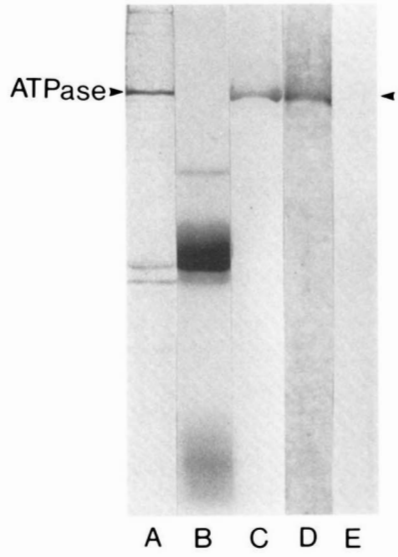

FIG. 3. **Immunoprecipitation of the (Ca^{2+} + Mg^{2+})-dependent ATPase from a [^{35}S]methionine-labeled extract of L6 myotubes.** *A*, autoradiogram of the immunoprecipitate obtained after incubation of a [^{35}S]methionine-labeled extract of L6 myotubes with 10 μl of the antiserum prepared against the (Ca^{2+} + Mg^{2+})-dependent ATPase. *B*, Coomassie blue stain of *gel A*. *C*, purified rat ATPase (Coomassie blue stain). *D*, radioiodinated rabbit ATPase immunoprecipitated with the same antiserum. *E*, autoradiogram of the second immunoprecipitate obtained from the cellular extract with an additional 10 μl of the antiserum. SDS-polyacrylamide gel electrophoresis in an 8.5% Laemmli slab gel was carried out in the presence of 2-mercaptoethanol.

sarcoplasmic reticulum proteins treated with the antiserum prepared to the 53,000-Da glycoprotein and incubated with radioiodinated protein A. The figure shows that two glycoproteins of molecular weights 53,000 and 160,000, present in sarcoplasmic reticulum membrane, cross-reacted with the antiserum. Since the antibodies bound to the proteins that were separated electrophoretically, the immunoprecipitation of the 160,000-Da glycoprotein with the 53,000-Da glycoprotein could not have been caused merely by aggregation between these two glycoproteins.

The antiserum prepared in rabbit against the (Ca^{2+} + Mg^{2+})-dependent ATPase from rat sarcoplasmic reticulum reacted with a 105,000-Da protein in the Triton extract of [^{35}S]methionine-labeled L6 myotubes. Fig. 3*B* shows a typical protein pattern of the immunoprecipitate and Fig. 3*A* shows a typical autoradiogram of the immunoprecipitate. The molecular weight of the protein synthesized in L6 myotubes corresponded to the molecular weight of the purified rat (Ca^{2+} + Mg^{2+})-dependent ATPase (Fig. 3*C*) and to the molecular weight of the radioiodinated rabbit ATPase, immunoprecipitated with the antibody (Fig. 3*D*). On the basis of radioactivity found in the gels of the first and the second immunoprecipitates in the region of the ATPase, we have calculated that 95% of the newly synthesized protein was recovered in the first immunoprecipitate. The autoradiogram of the second immunoprecipitate is shown in Fig. 3*E*. The results indicate that the main intrinsic proteins of the sarcoplasmic reticulum membrane, the (Ca^{2+} + Mg^{2+})-dependent ATPase, and the 53,000- and 160,000-Da glycoproteins were present in L6 myotubes.

Localization of Sarcoplasmic Reticulum Proteins in Microsomal Fraction Isolated from L6 Myotubes—Microsomal and cytosolic fractions were obtained from homogenates of [^{35}S]methionine-labeled 8-day-old L6 myotubes and immunoprecipitation was used to isolate sarcoplasmic reticulum proteins from both fractions. Fig. 4, *A* and *B*, shows that the

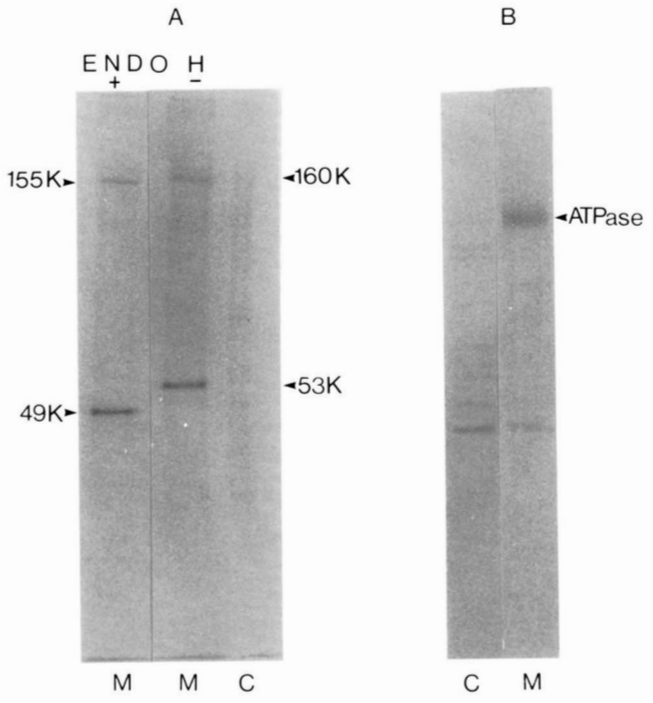

FIG. 4. **Localization of sarcoplasmic reticulum proteins in microsomal fractions obtained from L6 myotubes.** The microsomal and cytosolic fractions were obtained from [^{35}S]methionine-labeled myotubes of L6 as described under "Materials and Methods." Immunoprecipitates from the detergent extract of the microsomal and cytosolic fractions were formed with the antiserum prepared against the 53,000-Da glycoprotein (*A*) or ATPase (*B*) and isolated as described under "Materials and Methods." Endo H treatment of the immunoprecipitates was carried out as described under "Materials and Methods." *M*, immunoprecipitates obtained from the detergent extract of microsomal fraction; *C*, immunoprecipitates obtained from the cytosolic fractions.

53,000- and 160,000-Da glycoproteins and the ATPase were present in the microsomal fraction but were absent from the cytosol. This suggests that synthesis and processing of these proteins occurred in intracellular membranes.

Effect of Endo H on the 53,000- and 160,000-Da Glycoproteins—Treatment of the immunoprecipitates with Endo H, the enzyme which cleaves asparagine-linked di-*N*-acetylchitobiose sugar moieties from glycoproteins (22), caused reduction of the molecular weight of both the 53,000- and 160,000-Da proteins (Fig. 4*A*). This experiment shows that both polypeptides synthesized in L6 were glycoproteins similar to those found in the sarcoplasmic reticulum vesicles obtained from rabbit muscle (5).

Rates of Total Protein Synthesis during Differentiation of L6—L6 myoblasts growing in culture with an initial density of 10^6 cells/tissue culture flask multiplied between days 1 and 4 until they reached a confluent monolayer and then fused into multinucleated myotubes. Fig. 5 shows the rates of synthesis of the total protein extracted from the cells with Triton X-100 at different stages of development. The rate of total protein synthesis, expressed as counts per min/mg of total protein, was high and increased more during the initial period of cell division. However, at the time of intense cell fusion, total protein synthesis decreased sharply and remained at a constant level in the myotubes.

Patterns of Biosynthesis of Sarcoplasmic Reticulum Proteins during Differentiation of L6—The relative rates of biosynthesis of the 53,000- and 160,000-Da glycoproteins and the (Ca^{2+} + Mg^{2+})-dependent ATPase were measured at different time intervals during growth and differentiation of L6. Fig. 6, *A* and *B*, shows representative autoradiograms of the immunoprecipitates obtained with the antiserum prepared against the 53,000-Da glycoprotein and the ATPase, respectively, at different stages of development. The three intrinsic sarcoplasmic reticulum proteins were synthesized in L6 in a similar temporal pattern, even though two of them were glycosylated and one was not. When protein bands identified as the 53,000- and 160,000-Da glycoproteins and the ATPase were cut out of the gel, dissolved in hydrogen peroxide, and

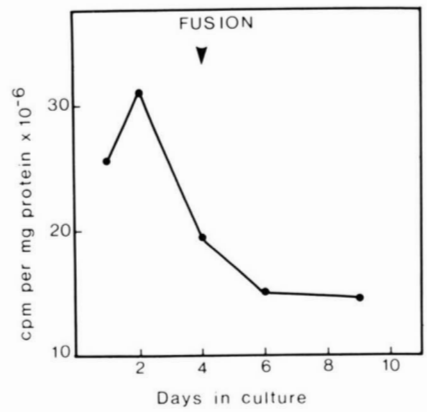

FIG. 5. **Rates of incorporation of [^{35}S]methionine into total cellular protein at different times during the differentiation of L6.** Cultures were labeled for 4 h with [^{35}S]methionine at the indicated time intervals after plating. Incorporation of [^{35}S]methionine into the total cellular protein was measured by the filter paper disc method (20).

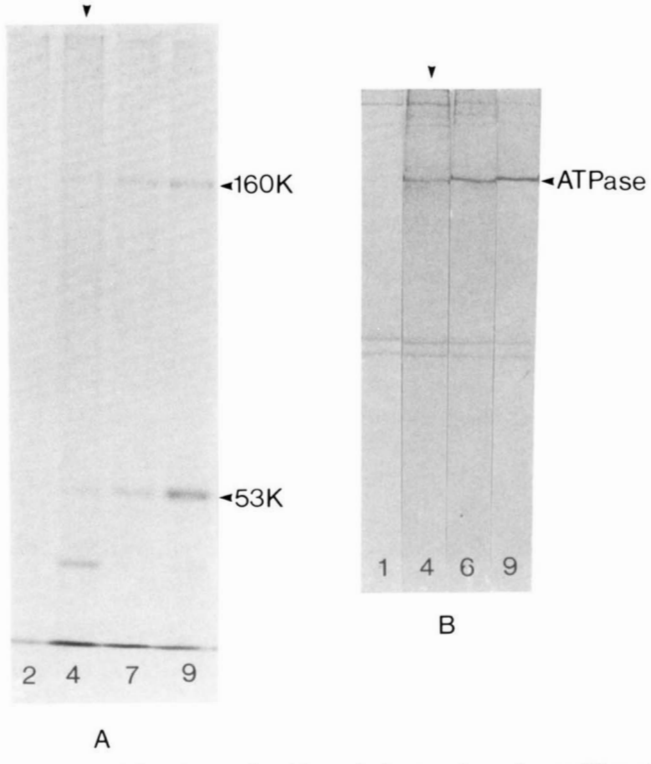

FIG. 6. **SDS-polyacrylamide gel electrophoretic profiles of immunoprecipitates obtained from differentiating L6.** Sarcoplasmic reticulum glycoproteins (*A*) or ATPase (*B*) were immunoprecipitated from Triton extracts of cells labeled with [^{35}S]methionine at the stages of differentiation indicated. At each point, 200 μg and 250 μg of cellular protein were used for immunoprecipitation with the antibody prepared against the 53,000-Da glycoprotein and the ATPase, respectively. The figure represents autoradiograms of the immunoprecipitates separated in 8.5% SDS-polyacrylamide slab gels prepared according to the method of Laemmli (16). The beginning of cell fusion is marked with *arrows*.

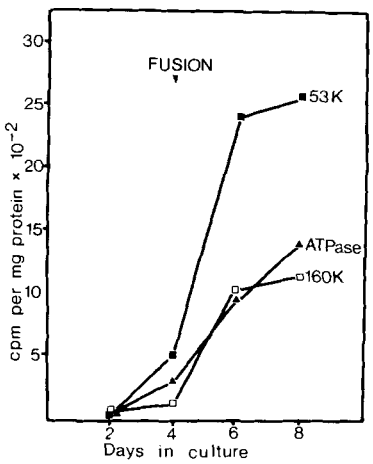

FIG. 7. **Rates of synthesis of intrinsic sarcoplasmic reticulum proteins during differentiation of L6.** Radioactivity incorporated into the sarcoplasmic reticulum glycoproteins or the ATPase was measured at the indicated time after plating as described under "Materials and Methods." At each point, 200 μg of cellular protein were taken for immunoprecipitation.

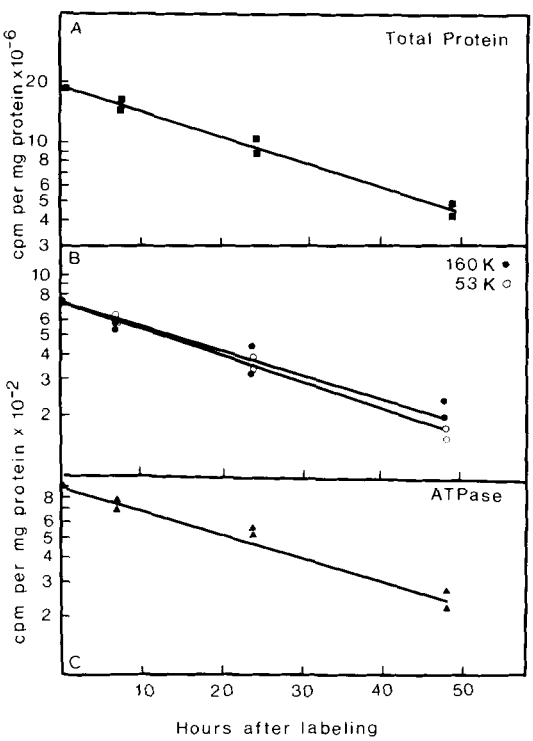

FIG. 8. **Rates of protein degradation in L6 myotubes.** Myotubes of L6 were incubated for 24 h in medium containing [^{35}S] methionine and 25% of the normal, unlabeled methionine content. The labeling medium was replaced with standard medium and cells were collected at the times indicated and analyzed for radioactivity remaining in total protein or intrinsic sarcoplasmic reticulum proteins as described under "Materials and Methods."

counted, protein synthesis was scarcely detectable in the mononucleated myoblasts. Biosynthesis of these three proteins was turned on at the beginning of fusion and rates of synthesis increased dramatically thereafter (Fig. 7). This occurred in spite of the fact that the rate of total protein synthesis decreased sharply after fusion began (Fig. 5). These results show that the time of biosynthesis of the intrinsic sarcoplasmic reticulum proteins is closely associated with the time of cell fusion and differentiation of myotubes.

Rates of Degradation of Sarcoplasmic Reticulum Proteins in L6 Myotubes—The rate of loss of radioactivity from total protein and from the sarcoplasmic reticulum proteins in the myotubes, following replacement of the labeling medium with medium that did not contain a radioactive amino acid, is presented in Fig. 8. In 8-day-old cultures, the 53,000- and the 160,000-Da glycoproteins and the (Ca^{2+} + Mg^{2+})-dependent ATPase turned over with a half-life of about 24 h. These half-lives were identical with the half-life of total cellular protein (Fig. 8).

DISCUSSION

It has recently been shown that sarcoplasmic reticulum membranes contain several glycosylated proteins including calsequestrin (5). Two of these glycoproteins have molecular weights of 53,000 and 160,000. These proteins show a number of similarities (5). They are both intrinsic membrane proteins, they both bind tightly to concanavalin A, both are Endo H-sensitive, they fractionate together on DEAE, and they react strongly with an antiserum raised against a sample of the 53,000-Da glycoprotein that had been excised from a slab gel and did not contain any high molecular weight protein. These similarities might suggest that the 160,000-Da glycoprotein is a trimer of the 53,000-Da glycoprotein, but we have not been able to obtain any evidence for subunit structure in the 160,000-Da glycoprotein. As a point of difference, the 53,000-Da glycoprotein stains pink with Stains All whereas the 160,000-Da glycoprotein stains dark blue (5). Present evidence suggests that both glycoproteins are unique components of the sarcoplasmic reticulum membrane but their cross-reactivity suggests that they share some amino acid sequence homology.

Cultures of embryonic muscle cells represent a favorable experimental system for investigating the biochemical events during cellular differentiation. In a series of previous studies of the assembly of the sarcoplasmic reticulum membrane in primary cultures of rat skeletal muscle, we have shown that two extrinsic proteins of the sarcoplasmic reticulum (calsequestrin and the high affinity calcium binding protein) are synthesized in a similar manner, even though one of them is glycosylated and the other is not (9–11). However, these proteins were not synthesized coordinately with the main intrinsic sarcoplasmic reticulum protein, the (Ca^{2+} + Mg^{2+})-dependent ATPase. Initiation of synthesis of the ATPase, which took place at about the same time as the beginning of cell fusion, was delayed for about 20 h, when compared with synthesis of the extrinsic proteins (8–11). Similar observations were reported for primary cultures of chicken myoblasts (23).

In this study, we have investigated temporal patterns of biosynthesis and degradation of the intrinsic glycoproteins of the sarcoplasmic reticulum and compared them with the temporal patterns of biosynthesis and degradation of the ATPase. Since both glycoproteins present in the [^{35}S]methionine-labeled extracts of L6 myotubes were precipitated with the antiserum prepared against the 53,000-Da glycoprotein, we were able to obtain concurrent information for both proteins. As a model of differentiating muscle, we have used the myogenic, continuous cell line L6. We have found that the pattern of synthesis and degradation of both glycoproteins was identical in the differentiating cells and indistinguishable from the pattern of synthesis and degradation of the ATPase. These results suggest that the intrinsic sarcoplasmic reticulum proteins are synthesized and turned over in a coordinated manner, regardless of whether they are glycosylated or not.

Results presented in this paper show that newly synthesized sarcoplasmic reticulum proteins are incorporated into membranes. Their presence in the microsomal fraction suggests that they were synthesized on bound polyribosomes and then

incorporated into the sarcoplasmic reticulum membrane. This confirms previous reports (24–26) showing the biosynthesis of the (Ca^{2+} + Mg^{2+})-dependent ATPase on bound polyribosomes.

The relationship between the synthesis of myofibrils and the assembly of the sarcoplasmic reticulum membrane is not well understood. Walker *et al.* (27) have suggested that the sarcoplasmic reticulum membrane provides a framework for the formation of myofibrils in embryonic muscle of human and monkey. By contrast, Fischman (28, 29) presented evidence that myofibrils observed at early stages of development in chicken muscle lacked a surrounding smooth membrane system. This could indicate that the sarcotubular system does not play a role in the assembly of myofibrils, but rather that myofibrils might provide a skeleton around which the membrane network is elaborated. On the basis of morphological studies performed on rabbit muscle differentiating *in vivo*, Kilarski and Jakubowska (30) suggested that myofibril formation and the assembly of the sarcoplasmic reticulum occur simultaneously in different cell compartments.

This paper presents evidence that the intrinsic sarcoplasmic reticulum proteins are not synthesized in dividing myoblasts but are synthesized at increasing rates in differentiating myotubes of L6. At the time when their rates of synthesis increased sharply, the rate of synthesis of bulk cellular protein decreased. Similar observations have been made in primary cultures of rat skeletal muscle (8). Since myofibrillar proteins represent the major fraction of total muscle protein (31), it is reasonable to assume that the intensive synthesis of these proteins and the beginning of myofibril formation precedes synthesis of the sarcoplasmic reticulum proteins and membrane assembly. In fact, Rogers *et al.* (32) demonstrated that in cultures of L6 the rate of synthesis of the heavy chain of myosin decreases after day 6.

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REFERENCES

1. Ebashi, S., Endo, M., and Ohtsuki, J. (1969) *Q. Rev. Biophys.* **2,** 351–384
2. Hasselbach, W. (1964) *Prog. Biophys. Mol. Biol.* **14,** 167–222
3. MacLennan, D. H., Yip, C. C., Iles, G. H., and Seeman, P. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37,** 469–477
4. MacLennan, D. H. (1970) *J. Biol. Chem.* **245,** 4508–4518
5. Campbell, K. P., and MacLennan, D. H. (1981) *J. Biol. Chem.* **256,** 4626–4632
6. MacLennan, D. H., and Wong, P. T. S. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 1231–1235
7. Ostwald, T. J., and MacLennan, D. H. (1974) *J. Biol. Chem.* **249,** 974–979
8. Holland, P. C., and MacLennan, D. H. (1976) *J. Biol. Chem.* **251,** 2030–2036
9. Zubrzycka, E., and MacLennan, D. H. (1976) *J. Biol. Chem.* **251,** 7733–7738
10. Michalak, M., and MacLennan, D. H. (1980) *J. Biol. Chem.* **255,** 1327–1334
11. Jorgensen, A. O., Kalnins, V. L., Zubrzycka, E., and MacLennan, D. H. (1977) *J. Cell Biol.* **74,** 287–298
12. Yaffe, D. (1968) *Proc. Natl. Acad. Sci. U. S. A.* **61,** 477–483
13. Ng, S. K., Rogers, J., and Sanwal, B. D. (1976) *J. Cell Physiol.* **90,** 361–374
14. Campbell, K. P., and MacLennan, D. H. (1982) *J. Biol. Chem.* **257,** 1238–1246
15. Branton, P. E., Lassam, N. J., Downey, J. F., Yee, S. I., Graham, F. L., Mak, S., and Bayley, S. T. (1981) *J. Virol.* **37,** 601–608
16. Laemmli, U. K. (1970) *Nature (Lond.),* **227,** 680–685
17. Habener, J. F., Rosenblatt, M., Dee, P. C., and Potts, J. T., Jr. (1979) *J. Biol. Chem.* **254,** 10596–10599
18. Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963) *Biochem. J.* **89,** 114–123
19. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
20. Mans, R. Y., and Novelli, D. (1961) *Arch. Biochem. Biophys.* **94,** 48–53
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
22. Tai, T., Yamashita, K., and Kobata, A. (1977) *Biochem. Biophys. Res. Commun.* **78,** 434–441
23. Ha, D-B., Boland, R., and Martonosi, A. (1979) *Biochim. Biophys. Acta* **585,** 165–187
24. Greenway, D. C., and MacLennan, D. H. (1978) *Can. J. Biochem.* **56,** 452–456
25. Chyn, T. L., Martonosi, A. N., Morimoto, T., and Sabatini, D. D. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 1241–1245
26. Reithmeier, R. A. F., de Leon, S., and MacLennan, D. H. (1980) *J. Biol. Chem.* **255,** 11839–11846
27. Walker, S. M., Schrodt, G. R., Currier, G. J., and Turner, E. V. (1975) *J. Morphol.* **146,** 97–128
28. Fischman, D. A. (1970) *Curr. Top. Dev. Biol.* **5,** 235–280
29. Fischman, D. A. (1972) in *The Structure and Function of Muscle* (Bourne, G. H., ed) 2nd Ed, Vol. I, pp. 75–148, Academic Press, New York
30. Kilarski, W., and Jakubowska, M. (1979) *Z. Microsk.-Anat. Forsch. (Leipz.)* **93,** 1159–1181
31. Benoff, S., and Nadal-Ginard, B. (1980) *J. Mol. Biol.* **140,** 283–298
32. Rogers, J., Coulter, M., Ng, S. K., and Sanwal, B. D. (1978) *Somatic Cell Genet.* **4,** 573–585