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\(^{2+} + \text{Mg}^{2+}\))-dependent ATPase in the differentiating rat myogenic continuous cell line, L6. Cells were labeled at various stages of differentiation with \(^{35}\text{S}\)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\(^{2+} + \text{Mg}^{2+}\))-dependent ATPase (4), a proteolipid (5), 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\(^{2+} + \text{Mg}^{2+}\))-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\(^{2+} + \text{Mg}^{2+}\))-dependent ATPase occurs in a coordinated manner during differentiation of L6.
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pH 7.5, 150 mM NaCl, and 100 KIU 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 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 of Trasylol as described earlier (14, 15). In separate experiments with bound immunoprecipitates obtained by centrifugation and washing five times with 1 ml of a solution containing 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 gel electrophoresis in 8.5% Laemmli gels (16) was carried out for 16 h at 30 mA (600 V) with 2-mercaptoethanol. The gels 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 50 μl of 0.1 M NaOH, and counting gel sections in a liquid 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 cellular 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 boiling, the SDS concentration was reduced by dialysis with 200 μl of 50 mM sodium citrate, pH 5.5. Samples containing 1 mg of protein were incubated for 16 h at 37 °C with 10 μl of Endo H according to the method of Gotoh and Sambrook (20). The reactions were stopped after 15 min by the addition of 5 μl of 0.1 M sodium metabisulfite 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, 10% dimethyl sulfoxide, 1 ml of Na2HPO4, and 0.05% chloramphenicol. The reaction was stopped after 15 min by the addition of 5 μl of 0.1 M sodium metabisulfite 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, and 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 radiiodinated protein A (500 cpm/ml). The blots were then 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 [35S]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 [35S]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 contrast microscope. The cell homogenate was centrifuged sequentially for 15 min at 10,000 × g and for 1 h at 100,000 × g.

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 (Ca2+ + Mg2+)-dependent ATPase—The (Ca2+ + Mg2+)-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. 1A shows the protein bands of a sample of a rat skeletal muscle extract with 100,000 × g supernatant containing 250 μg of protein.

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

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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, immunoprecipitated with protein A beads after incubation of a [35S]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 radiiodinated rabbit sarcoplasmic reticulum proteins and antiserum against the 53,000-Da glycoprotein. E, similar to lane C. F, autoradiogram of the immunoprecipitate obtained from a [35S]methionine-labeled cellular extract in D with an additional 20 μl of antiserum. G, autoradiogram of the immunoprecipitate obtained from a [35S]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-acetylgalactosami-
The molecular weight of this glycoprotein was very close to that of the 53,000-Da glycoprotein of sarcoplasmic reticulum. An immunoprecipitate obtained with the antibody prepared against the 53,000-Da glycoprotein of sarcoplasmic reticulum was used as a standard for identification of sarcoplasmic reticulum proteins in the absence of reducing agents. Fig. 1C is an autoradiogram which shows that the 53,000- and 160,000-Da proteins in the immunoprecipitate and the protein standards (Fig. 1A) ran in an identical position when gel electrophoresis was performed in the absence of reducing agents.

Fig. 1D shows that the antisera 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 antisera was prepared in sheep against the rabbit glycoprotein, it cross-reacted with rat proteins. As shown in Fig. 1, C and E, the antisera caused precipitation of both the 53,000- and the 160,000-Da glycoproteins from the Triton extract of "35S)methionine-labeled L6 myotubes. The antisera did not react with the 105,000-Da (Ca2+ + Mg2+)-dependent ATPase nor with the 63,000-Da calquearrin (Fig. 1, C, D and E). Fig. 1F 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. 1G). 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. 1G).

Fig. 2 is an autoradiogram of a nitrocellulose blot of rabbit sarcoplasmic reticulum proteins treated with the antisera prepared to the 53,000-Da glycoprotein and incubated with radiiodinated 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 antisera. 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 antisera prepared in rabbit against the (Ca2+ + Mg2+)-dependent ATPase from rat sarcoplasmic reticulum reacted with a 105,000-Da protein in the Triton extract of "35S)methionine-labeled L6 myotubes. Fig. 3B shows a typical protein pattern of the immunoprecipitate and Fig. 3A 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 (Ca2+ + Mg2+)-dependent ATPase (Fig. 3C) and to the molecular weight of the radiiodinated rabbit ATPase, immunoprecipitated with the antibody (Fig. 3D). 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. 3E. The results indicate that the main intrinsic proteins of the sarcoplasmic reticulum membrane, the (Ca2+ + Mg2+)-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 "35S)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...
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Fig. 4. Localization of sarcoplasmic reticulum proteins in microsomal fractions obtained from L6 myotubes. The microsomal and cytosolic fractions were obtained from [35S]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-acetylchitotobiose sugar moieties from glycoproteins (22), caused reduction of the molecular weight of both the 53,000- and 160,000-Da proteins (Fig. 4A). 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 10⁶ 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 (Ca²⁺ + Mg²⁺)-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...
Results show that the time of biosynthesis of the intrinsic proteins decreased sharply after fusion began (Fig. 5). These sarcoplasmic reticulum proteins is closely associated with the synthesis increased dramatically thereafter (Fig. 7). This occurred in spite of the fact that the rate of total protein synthesis was turned on at the beginning of fusion and rates of synthesis increased in mononucleated myoblasts. Biosynthesis of these three proteins occurred in spite of the fact that the rate of total protein remained in total protein.

Counts, 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 intracellular 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 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 intracellular 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 (35S)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\textsuperscript{2+} + Mg\textsuperscript{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.

Acknowledgments—We would like to thank Dr. Stephen Ng for his kind gift of the continuous myogenic cell line L6. The expert technical assistance of Kazimierz Kurzydlowski and Jerry Schulman is gratefully acknowledged.

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