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Correlation between fusion and the developmental regulation of membrane glycoproteins in L_6 myoblasts

(myoblast fusion/metalloendoprotease/fusion inhibitors)

JAY ROSENBERG, ALEX SZABO, DARYL RHEUARK, AND CELIK KAYALAR*

Department of Chemistry, University of California, Berkeley, CA 94720

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ABSTRACT **Expression of membrane glycoproteins in L_6 myoblasts during the course of myogenesis was investigated. The effects of several inhibitors of myoblast fusion and differentiation were also studied. The predominant change in plasma membrane proteins concomitant with fusion was the reduction in the expression of a major 105-kDa glycoprotein and the appearance of a 90-kDa glycoprotein. This change was blocked by bromodeoxyuridine and two metalloendoprotease inhibitors, phenanthroline and benzyloxycarbonyl-Ser-Leu-NH_2, all of which have been shown to inhibit myoblast fusion. The nature of this inhibition suggests a role for an endogenous metalloendoprotease in myoblast commitment to terminal differentiation. The possible function of the developmentally regulated glycoproteins in myogenesis is also discussed.**

During skeletal muscle development, mononucleate myoblasts proliferate, align, adhere, and fuse to form multinucleate myotubes. Tissue culture studies with established cell lines and primary cells have shown that myoblasts withdraw from the cell cycle at confluency and irreversibly commit to terminal differentiation (1). This process involves the fusion of myoblasts and the synthesis of muscle-specific proteins, such as myosin, creatine kinase, and acetylcholine receptors (1–3). Little is known about the molecular nature of the commitment process or the mechanisms of fusion and differentiation.

There is convincing evidence to indicate that membrane glycoproteins mediate the adhesion and fusion of myoblasts (4, 5). However, no protein that plays a direct role in either of these processes has been identified. One strategy for the identification of these proteins is to raise monoclonal antibodies against the myoblast surface and then to screen them for their inhibition of cell–cell adhesion or fusion (or both). So far this approach has yielded antibodies that inhibit only cell–substratum adhesion (6).

Another approach that several laboratories have undertaken is to examine the changes in myoblast membrane proteins during myogenesis. The effect of BrdUrd, a potent inhibitor of myoblast fusion and differentiation (7), has also been studied. Only minor changes in membrane proteins have been reported and no good correlation with the adhesion or fusion process has been found (5, 8, 9).

Recent studies by Couch and Strittmatter (10, 11) indicate that an endogenous metalloendoprotease plays a role in myoblast fusion. Inhibition of the metalloendoprotease by various inhibitors was shown to correlate with the inhibition of myoblast fusion in primary rat myoblast cultures as well as in the rat cell line L_6. These inhibitors included 1,10-phenanthroline (a divalent cation chelator with a high affinity for zinc) and benzyloxycarbonyl-Ser-Leu-NH_2 (Cbz-Ser-Leu-NH_2) (a synthetic peptide that competes with substrates of various metalloendoproteases). These inhibitors were also shown to inhibit the endogenous metalloendoprotease activity in the cell extracts of L_6 myoblasts (11). The cellular target(s) of this endogenous metalloendoprotease has not been identified. In addition, although these authors have clearly documented the involvement of a metalloendoprotease in myoblast fusion, they have not investigated the possibility that the metalloendoprotease might actually be involved in the initiation of myoblast commitment to terminal differentiation.

The present study has two objectives: (*i*) to investigate the expression of plasma membrane glycoproteins of L_6 myoblasts during myogenesis and (*ii*) to study the effects of certain inhibitors of myoblast fusion or differentiation (or both) on the expression of these glycoproteins.

MATERIALS AND METHODS

Cells and Cell Culture. A highly myogenic subclone of the rat myoblast cell line L_6, first isolated by Yaffe (12), was used. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 0.1% glucose, 10% fetal calf serum, and 100 units of penicillin/streptomycin (Cell Culture Facility, University of California, San Francisco) per ml and were incubated at 37°C in 8% CO_2 in air. Cells were plated at an initial density of 5.5×10^5 cells in Falcon 150-mm dishes containing 40 ml of medium. The medium was changed every 48 hr. In BrdUrd-treated cultures, 6.4 μM BrdUrd (Sigma) was added 24 hr after plating and was included in the medium thereafter.

Labeling of Cells with *N*-Acetyl[^{3}H]glucosamine and [^{3}H]Mannose. Cells to be labeled were gently washed twice with 20 ml of DMEM containing 0.01% glucose. Fifteen milliliters of this same medium supplemented with 100 μCi (1 Ci = 37 GBq) each of *N*-acetyl-D-[6-^{3}H]glucosamine hydrochloride (Amersham) (40 Ci/mmol) and D-[2-^{3}H]mannose (Amersham) (10–20 Ci/mmol) was then added. In fusion-inhibited preparations, BrdUrd or Cbz-Ser-Leu-NH_2 (Vega Biochemicals, Tucson, AZ) was also present during labeling. After a 17-hr incubation at 37°C in 8% CO_2, the labeling medium was removed and the cells were washed twice with 10 ml of ice-cold phosphate-buffered saline (P_i/NaCl). Cells were harvested by scraping with a rubber policeman in the presence of 5 ml of ice-cold P_i/NaCl.

Preparation of Plasma Membranes. Plasma membranes were prepared essentially as described by Schimmel *et al.* (13). Purity of different membrane fractions were determined by assaying for marker enzymes (13).

Gel Electrophoresis and Autoradiography. $NaDodSO_4$/PAGE was performed with 1.5-mm-thick slab gels (3% stacking gel and 5–10% continuous-gradient separating gel) with the discontinuous buffer system of Laemmli (14). Gels

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Abbreviation: Cbz-Ser-Leu-NH_2, benzyloxycarbonyl-Ser-Leu-NH_2.

*To whom reprint requests should be addressed.

were fixed with 10% acetic acid and treated with EN-^{3}HANCE (New England Nuclear). After drying, gels were autoradiographed by using Kodak X-Omat film.

Assay of Fusion. Determination of percentage of myoblast fusion was essentially as described by Couch and Strittmatter (10). Fusion was quantified from photographs of culture plates by phase-contrast microscopy. The number of myotubes per 0.5 mm^2 of culture plate was counted and averaged. The presence of more than one nucleus per cell, branching of the cell contour, and cell size of about three times that of myoblasts were used as criteria for fusion (2). The level of fusion in untreated, control cultures at stage 3 (168 hr after plating) was taken as 100%.

RESULTS

Expression of Plasma Membrane Glycoproteins During Myogenesis. We have studied the synthesis of plasma membrane glycoproteins in the L_6 cell line at three different stages of myogenesis: stage 1 (sparse myoblasts, 48 hr after plating) (Fig. 1 *Left*), stage 2 (confluent, midfusion myoblasts, 120 hr after plating) (Fig. 1 *Center*), and stage 3 (myotubes, 168 hr after plating) (Fig. 1 *Right*). Cells were labeled with *N*-acetyl[^{3}H]glucosamine and [^{3}H]mannose for 17 hr starting at these three stages of differentiation. Plasma membranes were then prepared and subjected to NaDodSO$_4$/PAGE followed by autoradiography.

Typical results from a time-course labeling of plasma membranes are shown in Fig. 2. A major glycoprotein of 105 kDa is present in the plasma membrane of stage 1 myoblasts (lane a, arrow). At stage 2, labeling of this protein is significantly reduced. Another protein of about 90 kDa, which is hardly detectable in stage 1, appears in stage 2 as the major plasma membrane glycoprotein (lane b, arrow). Other differences in glycoprotein expression can also be observed between stages 1 and 2, but these are small by comparison. At stage 3, expression of the glycoproteins is similar to that at stage 2 (lane c).

An estimation of the magnitude of change in the 105- and 90-kDa glycoproteins was made by using the autoradiogram scans of Fig. 2 as well as by counting the radioactivity in the corresponding bands that were cut out of similar gels.

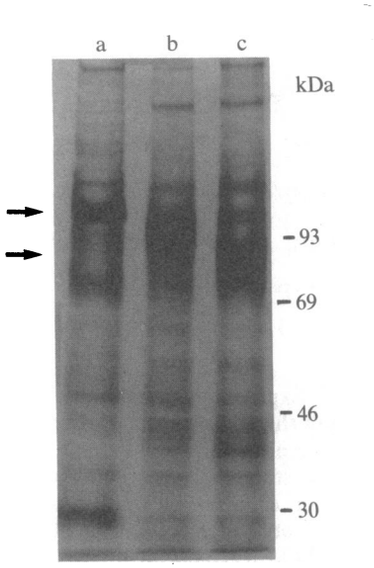

FIG. 2. *N*-acetyl[^{3}H]glucosamine and [^{3}H]mannose incorporation into plasma membrane glycoproteins at different stages of myogenesis. L_6 cells were labeled with *N*-acetyl[^{3}H]glucosamine and [^{3}H]mannose for 17 hr starting at each of the three stages of differentiation. Plasma membranes were then prepared from these cells and subjected to NaDodSO$_4$/PAGE followed by autoradiography. Lanes: a, stage 1 myoblasts; b, stage 2 myoblasts; c, stage 3 myoblasts.

Average ratios of the radioactivity in the 105-kDa band over the 90-kDa band were calculated [i.e., 105 kDa (cpm)/90 kDa (cpm)]. For the three stages of myogenesis, as in Fig. 2, these ratios were found to be >50, 0.36, and 0.19, respectively. We refer to the change in the expression of these two major glycoproteins as the "glycoprotein switch."

Treatment of the plasma membranes with saponin and high salt concentration did not affect the results shown in Fig. 2 (data not shown). This treatment has been shown to remove proteins loosely bound to the membrane surface or entrapped during membrane preparation (15). These results indicate that the 105- and 90-kDa glycoproteins are intrinsic membrane proteins.

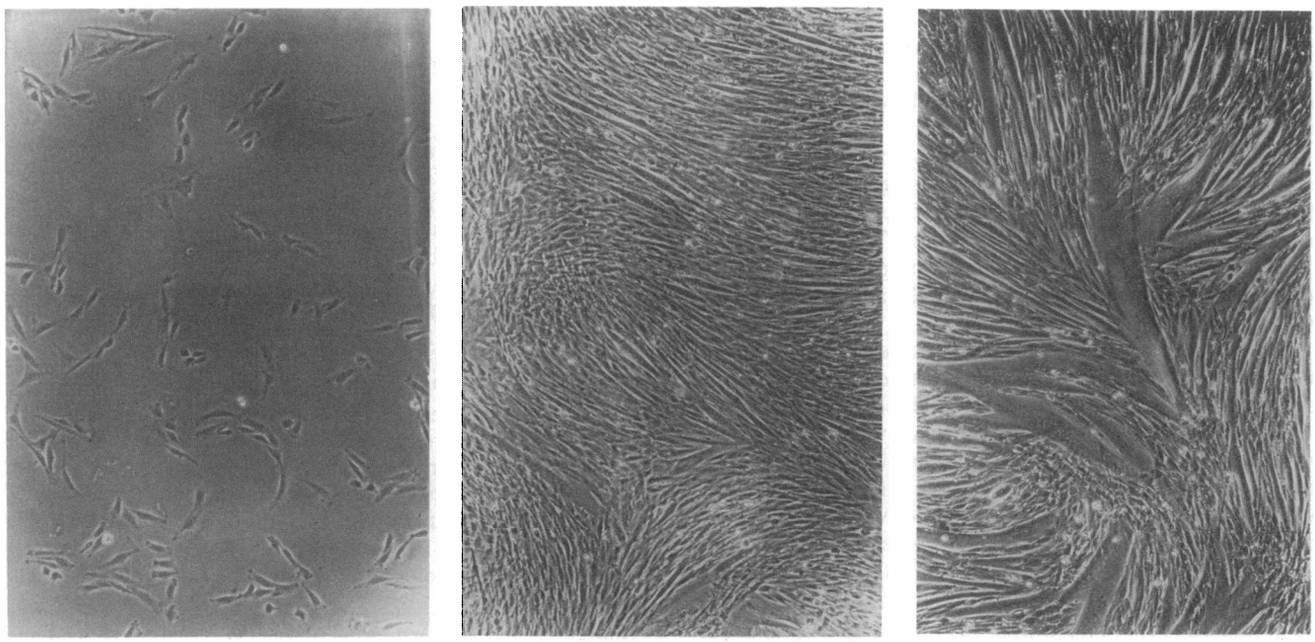

FIG. 1. Cultures of rat L_6 myoblasts at different stages of myogenesis. (*Left*) Stage 1 (sparse myoblasts, 48 hr after plating). (*Center*) Stage 2 (confluent, midfusion myoblasts, 120 hr). (*Right*) Stage 3 (myotubes, 168 hr).

Effect of BrdUrd on the Expression of Plasma Membrane Glycoproteins. BrdUrd at low concentrations inhibits the expression of cell-specific characteristics of several differentiating systems, including myoblasts (7). Sparse myoblasts treated with BrdUrd continue to proliferate normally but are inhibited in their commitment to terminal differentiation. Thus, these myoblasts neither fuse nor synthesize muscle-specific proteins, although they remain completely viable (16). Although the molecular basis of this effect is not fully understood, BrdUrd is believed to alter gene expression by incorporation into DNA during cell division (7).

To determine the effect of BrdUrd on glycoprotein expression, L_6 cells were grown in the presence of 6.4 μM BrdUrd and were labeled with *N*-acetyl[^{3}H]glucosamine and [^{3}H]-mannose at stages 2 and 3. NaDodSO$_4$/PAGE and autoradiography of these plasma membrane preparations revealed that the glycoprotein switch was indeed inhibited, regardless of culture age (Fig. 3). Thus, BrdUrd-treated cells exhibit a glycoprotein pattern similar to that of untreated cells at stage 1 (sparse myoblasts).

Effect of Metalloendoprotease Inhibitors on Expression of Plasma Membrane Glycoproteins. We investigated whether the metalloendoprotease inhibitors phenanthroline and Cbz-Ser-Leu-NH$_2$, which inhibit myoblast fusion (10), would also block the glycoprotein switch. In one set of experiments, confluent, unfused myoblasts (94 hr after plating) were incubated for 2 hr with growth medium containing 80 μg of phenanthroline per ml. After extensive washing, the cells were provided with their normal growth medium. L_6 cells treated in this manner remain unfused for up to 3 days, whereas untreated control myoblasts attain their maximal fusion level during this time period. Twenty-four hours after treatment (at stage 2) the myoblasts were labeled with *N*-acetyl[^{3}H]glucosamine and [^{3}H]mannose, and membranes were prepared and analyzed by NaDodSO$_4$/PAGE. As seen in Fig. 4 (lane b), the glycoprotein switch is inhibited in phenanthroline-treated myoblasts. Control cells treated with ethanol under the same conditions (lane a) undergo the glycoprotein switch and appear similar to untreated stage 2 myoblasts in Fig. 2 (lane b).

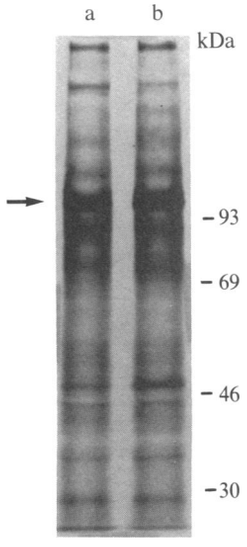

FIG. 3. *N*-acetyl[^{3}H]glucosamine and [^{3}H]mannose incorporation into plasma membrane glycoproteins of BrdUrd-treated cells. L_6 cells grown in medium containing 6.4 μM BrdUrd were labeled with *N*-acetyl[^{3}H]glucosamine and [^{3}H]mannose in the presence of this inhibitor for 17 hr at stages 2 and 3. Plasma membranes were then prepared from these cells and subjected to NaDodSO$_4$/PAGE followed by autoradiography. Lanes: a, stage 2 myoblasts; b, stage 3 myoblasts.

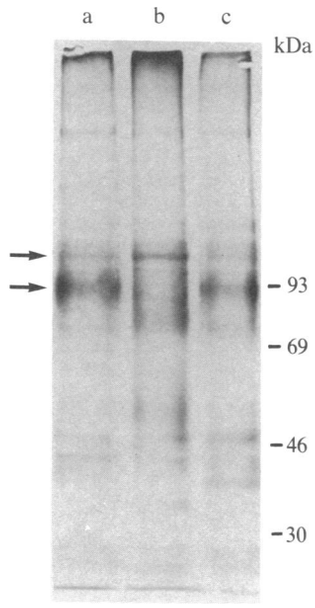

FIG. 4. *N*-acetyl[^{3}H]glucosamine and [^{3}H]mannose incorporation into plasma membrane glycoproteins of cells treated with 1,10-phenanthroline. Confluent, unfused L_6 myoblasts (96 hr after plating) were treated with growth medium containing either 1% ethanol (control cells) or 80 μg of phenanthroline per ml for 2 hr at 37°C. The cells were then washed twice and incubated in regular growth medium for an additional 24 hr. At this time (122 hr after plating), the cells were labeled with *N*-acetyl[^{3}H]glucosamine and [^{3}H]mannose for 17 hr, plasma membranes were prepared, and NaDodSO$_4$/PAGE and autoradiography were performed. In a parallel experiment, stage 2 myoblasts were treated with 80 μg of phenanthroline per ml for 2 hr just prior to labeling. Lanes: a, control cells; b, cells treated with phenanthroline at confluency; c, cells treated with phenanthroline at stage 2.

Inhibition of the glycoprotein switch by phenanthroline is consistent with a role for an endogenous metalloendoprotease in the switch process. To test this possibility further, the specific metalloendoprotease inhibitor Cbz-Ser-Leu-NH$_2$ was also used. Previous studies have shown that Cbz-Ser-Leu-NH$_2$ added to confluent, unfused myoblasts will completely inhibit fusion (10). This inhibition is reversible since removal of the dipeptide allows the cells to fuse extensively.

The effect of Cbz-Ser-Leu-NH$_2$ on the glycoprotein switch is shown in Fig. 5. In one set of experiments, 0.75 mM Cbz-Ser-Leu-NH$_2$ was added to the medium of confluent, unfused myoblasts. Twenty-four hours after this treatment (at stage 2) the cells were labeled with *N*-acetyl[^{3}H]glucosamine and [^{3}H]mannose in the presence of this inhibitor. As can be seen in Fig. 5 (lane b), the glycoprotein switch is inhibited in cells treated with the dipeptide in this manner. Moreover, these cells did not fuse as long as the dipeptide was present in the culture media. Control cells are shown in lane a.

These experiments indicate that when an endogenous metalloendoprotease of confluent myoblasts is inhibited before the onset of fusion, the glycoprotein switch and fusion are blocked. It is conceivable that the glycoprotein switch is due to the proteolytic cleavage of the 105-kDa glycoprotein by a metalloendoprotease to give the 90-kDa form. If this were the case, then treating myoblasts with these inhibitors after the onset of fusion should still inhibit the glycoprotein switch.

To address this possibility, midfusion myoblasts (stage 2) were treated for 2 hr with phenanthroline, washed extensively, and labeled for 17 hr with *N*-acetyl[^{3}H]glucosamine and [^{3}H]mannose. Results in Fig. 4 (lane c) show that the glycoprotein switch for the newly synthesized glycoproteins continues to occur if the inhibitor is added after cell fusion commences.

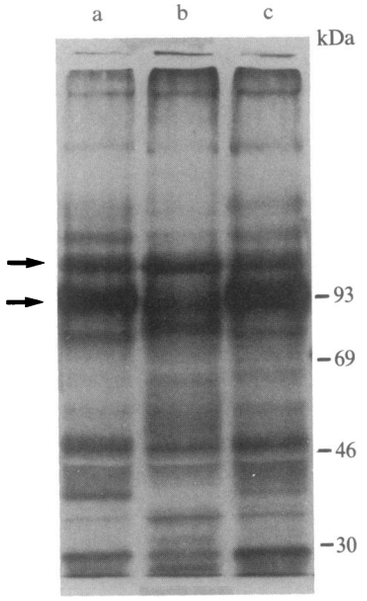

FIG. 5. *N*-acetyl[^{3}H]glucosamine and [^{3}H]mannose incorporation into plasma membrane glycoproteins of cells treated with the metalloendoprotease inhibitor Cbz-Ser-Leu-NH_2. Confluent, unfused L_6 myoblasts (96 hr after plating) were treated with growth medium containing either 1% methanol (control cells) or 0.75 mM Cbz-Ser-Leu-NH_2 for 24 hr at 37°C. The cells were then labeled with *N*-acetyl[^{3}H]glucosamine and [^{3}H]mannose in the presence of this inhibitor for 17 hr, plasma membranes were prepared, and $NaDodSO_4$/PAGE and autoradiography were performed. In a parallel experiment, 0.75 mM Cbz-Ser-Leu-NH_2 was added to the medium of stage 2 myoblasts exclusively during the 17-hr labeling period. Lanes: a, control cells; b, cells treated with Cbz-Ser-Leu-NH_2 since confluency; c, cells treated with Cbz-Ser-Leu-NH_2 since stage 2.

Myoblasts under these conditions continue to fuse, albeit at a slightly slower rate then the untreated controls.

In a comparable experiment, midfusion myoblasts were treated with 0.75 mM Cbz-Ser-Leu-NH_2 only during the 17-hr labeling period. As can be seen in Fig. 5 (lane c), the glycoprotein switch still occurs in these cells. Myoblasts treated in this manner continue to fuse at a slightly slower rate than the untreated controls.

A summary of the effects of these inhibitors on myoblast fusion is presented in Table 1.

DISCUSSION

In this investigation, we have studied the expression of plasma membrane glycoproteins of L_6 myoblasts during myogenesis. We have identified a dramatic change in the expression of two major glycoproteins that takes place at the onset of myoblast fusion. This change, which we have termed the glycoprotein switch, involves a drastic reduction in the expression of a 105-kDa glycoprotein and a simultaneous appearance of a 90-kDa glycoprotein.

Table 1. Effect of inhibitors on myoblast fusion

Treatment	Time of treatment,* hr after plating	% fusion† 120 hr	% fusion† 168 hr
Control	—	42 ± 4	100 ± 9
BrdUrd	24–168	<4	<4
1,10-Phenanthroline	94–96	<4	5 ± 3
	120–122	42 ± 4	91 ± 9
Cbz-Ser-Leu-NH_2	94–168	<4	<4
	120–168	42 ± 4	83 ± 9

*Ninety-four hours after plating, myoblasts were confluent but unfused. Phenanthroline treatments were for 2 hr. BrdUrd and Cbz-Ser-Leu-NH_2 treatments were continuous during time periods indicated.

†The level of fusion in control cultures at 168 hr after plating was taken as 100%.

Although others have labeled myoblast plasma membrane glycoproteins with various sugars, there have been no previous reports, to our knowledge, to indicate the regulation of expression of such major glycoproteins. We believe that our success in identifying these glycoproteins is due to a series of preliminary experiments in which we optimized the sugar-labeling conditions for maximal incorporation of radioactivity. Our conditions differ from others, mainly in the low level of glucose (0.01%) used during labeling, which seems to provide the highest specific activity of labeled sugars without affecting cell metabolism significantly.

In parallel experiments, when plasma membrane proteins were labeled with [^{35}S]methionine, a large number of proteins were observed after $NaDodSO_4$/PAGE and autoradiography. However, in contrast to sugar-labeling experiments, no significant change in the expression of these proteins could be detected at the three different stages of myogenesis studied (data not shown). Apparently, when all plasma membrane proteins were labeled with [^{35}S]methionine, the specific change in the 105- and 90-kDa glycoproteins was masked due to the presence of other proteins in that molecular mass range.

Myoblasts treated with specific metalloendoprotease inhibitors do not undergo the glycoprotein switch. This raises the possibility that the 105-kDa glycoprotein is cleaved by an endogenous metalloendoprotease to generate the 90-kDa form. However, these metalloendoprotease inhibitors have no effect on the glycoprotein switch if they are added subsequent to the onset of fusion. Thus, a direct role for the metalloendoprotease in the glycoprotein switch seems unlikely. It is still possible, however, that the 105-kDa glycoprotein is converted to the 90-kDa form through the action of another protease (which in turn might be activated by a metalloendoprotease). Further experiments are necessary to determine if there is a precursor–product relationship between these two glycoproteins.

The function of these glycoproteins is currently unknown. The glycoprotein switch occurs either simultaneously or shortly before any fusion can be observed. Furthermore, myoblast fusion and the glycoprotein switch are inhibited under the same conditions. Therefore, it is conceivable that the glycoprotein switch plays a direct role in the fusion process. For example, the 90-kDa glycoprotein could be a fusogenic protein whose appearance on the cell surface facilitates the fusion of two apposing myoblast membranes. This would be somewhat analogous to the proposed mechanism for the fusion of Sendai virus with its host cell. In that system, a viral membrane glycoprotein has been shown to be cleaved by a cellular protease to generate a fusogenic peptide (17). Purification of the 105- and 90-kDa glycoproteins and reconstitution of them into liposomes will allow testing of their possible fusogenic activities by using an *in vitro* fusion assay developed earlier (18). It is also possible, however, that the glycoprotein switch does not play any role in the fusion process but simply takes place concomitant with fusion as one of the many changes associated with terminal differentiation. If this were the case, then the glycoprotein switch can be considered a new marker of differentiation whose function is yet to be determined.

As mentioned earlier, the glycoprotein switch is inhibited only when myoblasts are treated with metalloendoprotease inhibitors prior to the onset of fusion. A plausible interpretation of this result is that an endogenous metalloendoprotease is required for the initiation of myoblast commitment to

terminal differentiation. Once the commitment occurs, subsequent inhibition of the metalloendoprotease affects neither the glycoprotein switch nor the progress of fusion significantly. In accordance with this interpretation, recent experiments performed in our laboratory show that phenanthroline treatment indeed inhibits the activation of the synthesis of myosin and creatine kinase (two typical markers of differentiation). This inhibition is most pronounced when myoblasts are treated with phenanthroline prior to the onset of fusion. Further work must be performed before the exact role of the metalloendoprotease and the glycoprotein switch in myogenesis can be determined.

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