

# The Calcium-dependent Myoblast Adhesion that Precedes Cell Fusion Is Mediated by Glycoproteins

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**ABSTRACT** Presumptive myoblasts from explants of chick embryo pectoral muscle proliferate, differentiate, and fuse to form multinucleate myotubes. One event critical to multinucleate cell formation is the specific adhesion of myoblasts before union of their membranes. In the studies reported here five known inhibitors of myotube formation—trifluoperazine, sodium butyrate, chloroquine, 1,10 phenanthroline, and tunicamycin—were tested for their effect on the  $\text{Ca}^{++}$ -dependent myoblast adhesion step. The first four inhibitors of myotube formation do not perturb myoblast adhesion but rather block fusion of aggregated cells, which suggests that these agents perturb molecular events required for the union of the lipid bilayers. By contrast, tunicamycin exerts its effect by inhibiting the myoblast adhesion step, thereby blocking myotube formation. The effect of tunicamycin can be blocked by a protease inhibitor, however, which implies that the carbohydrate residues protect the glycoproteins from proteolytic degradation rather than participate directly in cell-cell adhesion. Whereas trypsin treatment of myoblasts in the absence of  $\text{Ca}^{++}$  destroys the cells' ability to exhibit  $\text{Ca}^{++}$ -dependent adhesion, the presence of  $\text{Ca}^{++}$  during trypsin treatment inhibits the enzyme's effect, which suggests that myoblast adhesion is mediated by a glycoprotein(s) that has a conformation affected by  $\text{Ca}^{++}$ . Finally, myoblast adhesion is inhibited by an antiserum raised against fusion-competent myoblasts. The effect of the antiserum is blocked by a fraction from the detergent extract of pectoral muscle that binds to immobilized wheat germ agglutinin, which again suggests that glycoproteins mediate  $\text{Ca}^{++}$ -dependent myoblast adhesion.

Embryonic muscle can be excised from 11-d chicks, enzymatically dissociated, filtered, and grown in culture. The resulting presumptive myoblasts proliferate, differentiate, align in culture, and fuse to form multinucleate myotubes that express the biochemical and morphological characteristics of muscle (4, 7; reviewed in 3, 23, 46). This progression of orderly and timed events is accompanied by regulated expression of new molecules, migration of cells, adhesion of myoblasts, and union of cell membranes.

To shed light on the molecular nature of the events that occur during the formation of multinucleate cells, investigators have sought means to inhibit the easily recognizable event of myotube formation. Several agents and conditions that have been found to block myotube formation include EDTA (45), cytochalasin B (24), lectins (13), phospholipase C (38), lysolecithin (48), exogenously added cholesterol (57), inhibition of endogenous cholesterol synthesis in cells grown in lipid-depleted medium (8), and enrichment of elaidate (trans-

oleate) in the cellular membrane (25). More recently five other agents have been added to the growing list of inhibitors of myotube formation: trifluoperazine (TFP)<sup>1</sup> (1), an antagonist of the calcium-binding protein calmodulin (30, 36); chloroquine (28), a lysomotropic amine that inhibits the function of lysosomal enzymes (42) and alters the turnover of receptors (19, 29); 1,10 phenanthroline, a metal chelator that inhibits soluble metalloendoproteases in myoblasts (9, 10); sodium butyrate (15), a short chain fatty acid that may modify the cell membrane; and tunicamycin (18), an inhibitor of dolichol-phosphate-mediated glycosylation of proteins at asparagine-linked sites. Taken together the results of these numerous inhibitor studies have led investigators to the general conclusions that calcium, proteins and glycoproteins, lipids, and the cytoskeleton are all somehow involved in myoblast fusion.

<sup>1</sup> Abbreviations used in this paper: TFP, trifluoperazine; WGA, wheat germ agglutinin.

To understand a cellular process as complex as myogenesis on a molecular level, it is necessary to dissect it into steps that can be manipulated experimentally. One such step central to myogenesis is the recognition and adhesion of fusion-competent myoblasts. Previous work by Knudsen and Horwitz (32) showed that cultured myoblasts express an enhanced ability to adhere to one another in suspension at the same time that they express an optimal ability to fuse. The adhesion depends on  $\text{Ca}^{++}$  (as does myotube formation) and is specific (32). The existence of  $\text{Ca}^{++}$ -dependent myoblast adhesion and, in addition,  $\text{Ca}^{++}$ -independent adhesion has recently been confirmed by the work of Gibraltar and Turner (17). Earlier, Knudsen and Horwitz had shown that the  $\text{Ca}^{++}$ -dependent myoblast adhesion displays optima for pH, temperature,  $\text{Ca}^{++}$  concentration, and age of cell culture that closely parallel those for myotube formation (32). Inhibitor studies by the same authors showed that some agents able to inhibit myotube formation—such as energy poisons, trypsin, an inhibitor of cholesterol synthesis, and inhibitors of protein synthesis—block myoblast adhesion, whereas other inhibitors of myotube formation, such as cytochalasin B, colchicine, phospholipase C, or enrichment of membranes in elaidate, have no effect on myoblast adhesion (33). Taken together, these results led Knudsen and Horwitz to conclude that a separate, distinct adhesion event that depends on  $\text{Ca}^{++}$  and involves cell surface proteins precedes the fusion of myoblast membranes.

The studies reported here extend our understanding of the nature of the molecules involved in the specific,  $\text{Ca}^{++}$ -dependent myoblast adhesion step of myogenesis. A preliminary report of this work has been published in abstract form (31).

## MATERIALS AND METHODS

**Materials:** TFP, chloroquine (diphosphate salt), 1,10 phenanthroline, leupeptin, and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO). Tunicamycin was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Sodium butyrate was from J. T. Baker Chemical Co. (Phillipsburg, NJ). These reagents were stored according to the manufacturer's instructions and dissolved immediately before use, except for tunicamycin, which was stored at  $-70^{\circ}\text{C}$  as a  $500\ \mu\text{g}/\text{ml}$  stock solution in  $\text{H}_2\text{O}/\text{dimethyl sulfoxide}$ , 1:1. Optimal times of addition to the culture medium and optimal concentrations of the agents being tested were determined experimentally to be those that blocked the myotube formation initiated by adding  $\text{Ca}^{++}$  to 52-h EGTA-blocked cultures without causing cellular toxicity, as determined by morphological examination of the cells by phase-contrast microscopy and trypan blue exclusion.

**Cell Culture:** Primary cultures of pectoral muscle cells and fibroblasts were prepared from 11-d chick embryos as described (32). Myoblast cultures were plated on collagen-coated culture dishes at  $6 \times 10^6$  cells/100-mm dish and grown in  $\text{Ca}^{++}$ -free Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 1% Penn-Strep and 0.5% Fungizone (both from Flow Laboratories, Inc., McLean, VA), and 5 mM HEPES at  $37^{\circ}\text{C}$  in an atmosphere of 90% air/10%  $\text{CO}_2$ . EGTA was added to the cells at a concentration of 200–300  $\mu\text{M}$  to prevent myoblast fusion.

**Harvesting Myoblasts:** EGTA-blocked, fusion-competent myoblasts were detached from the substratum 50–51 h after plating by adding a heat-inactivated antiserum that specifically disrupts cell-substratum adhesion of myoblasts. This antiserum was raised in a rabbit against cell surface, integral membrane glycoproteins involved in cell-substratum adhesion (34) purified from chick cells by antibody affinity chromatography using a monoclonal antibody (CSAT) that rounds and detaches myoblasts from a variety of substrata but does not interfere with myoblast fusion (26, 40). A maximum effect in a reasonable amount of time resulted when the antiserum was added at dilutions of from 1:200 to 1:500 3 to 4 h before the aggregation assays. The rounded myoblasts were removed from the culture dish by gentle pipetting, concentrated by centrifugation, and resuspended in  $\text{Ca}^{++}$ -free Hanks' balanced salt solution containing 5% glucose, 10 mM HEPES, and, when indicated, 1 mM  $\text{Ca}^{++}$ .

## Production of Rabbit Antiserum against Fusion-Competent

**Myoblasts:** EGTA-blocked myoblasts were harvested 52 h after plating using the cell-substratum, adhesion-perturbing rabbit antiserum described above. Approximately  $10^7$  cells were washed with phosphate-buffer saline (PBS), pelleted by centrifugation, resuspended in 0.5 ml PBS, mixed 1:1 with either complete (first injection) or incomplete Freund's adjuvant, and injected subcutaneously in four sites in a rabbit. Four injections were given at 2-wk intervals before the rabbit was bled and the heat-inactivated serum was tested for its effect on myoblast aggregation and myotube formation.

**Assays for Myoblast Aggregation:** Myoblast aggregation was determined on 52-h cultures by two assays. In one assay, the cell concentration was adjusted to  $2.5\text{--}3.0 \times 10^5$  cells/ml, and 1-ml aliquots were mixed in 5-ml Fernbach flasks for 20 min at  $37^{\circ}\text{C}$  on an Adams Nutator (Clay Adams, Parsippany, NJ). At this cell concentration, the clusters that formed were small enough to permit counting of the aggregated myoblasts. The percentage of aggregated cells was scored by counting cells using phase-contrast microscopy as previously described (32).

In the second aggregation assay, the cell concentration was adjusted to  $1 \times 10^6$  cells/ml, and 0.5-ml aliquots were mixed in wells of a 24-well Costar dish (Costar, Cambridge, MA) at  $37^{\circ}\text{C}$  for 20 min on the Nutator. At this concentration, the cells formed large clusters composed of too many cells to count by microscopic observation. Instead, the results were recorded by photography. For both assays the inhibitors, when tested, were present during the incubation period.

**Assay for Myoblast Fusion:** To measure myotube formation,  $1\text{--}2$  mM  $\text{Ca}^{++}$  was added to EGTA-blocked cultures 50–52 h after plating, and fusion was quantitated 18–20 h later as previously described (32). To assess fusion of myoblasts aggregated in suspension, the cells mixed at a concentration of  $1 \times 10^6$  cells/ml for 20 min at  $37^{\circ}\text{C}$  were transferred to the wells of six-well Costar dishes (not collagen coated) and incubated in 2.5 ml of fresh medium overnight at  $37^{\circ}\text{C}$  in either the absence or presence of the inhibitors and/or  $\text{Ca}^{++}$ . No attempt was made to wash away any remaining cell-substratum adhesion-perturbing antibody. Fusion of cells in suspension, i.e., myoblast formation (33), was scored by microscopic observation using an inverted phase-contrast microscope and by recording the results photographically. Multinucleate cell formation was confirmed by exposing the cells to 0.1% trypsin and 1 mM EDTA in PBS for 15 min at  $37^{\circ}\text{C}$  and observing under the microscope whether or not this treatment dispersed the cells.

**Analysis of Protein Synthesis:** Protein synthesis was measured by trichloroacetic acid (TCA) precipitation of radiolabeled cells. 10–15  $\mu\text{Ci}$  of  $^3\text{H}$ -amino acid mix (New England Nuclear, Boston, MA) was added to 30-h EGTA-blocked cultures along with tunicamycin (0.1  $\mu\text{g}/\text{ml}$ ) or cycloheximide (5  $\mu\text{g}/\text{ml}$ ). 52 h after plating the cells, the medium was removed and centrifuged for 10 min at 15,000 rpm in an SS 34 rotor of a Sorvall RC2-B centrifuge (DuPont Instruments, Sorvall Biomedical Div., Wilmington, DE) to collect any cells that had detached from the substratum. Cold 10% TCA was added to the cells on the culture dish and to the few cells that were retrieved from the conditioned medium. The cells were scraped from the dishes with a rubber policeman and washed onto a 0.45- $\mu\text{m}$  filter under vacuum. The cells were collected and washed on the filters and the filters were monitored for radioactivity using the premixed liquid scintillation solution Formula 963 (New England Nuclear).

**Pectoral Muscle Extraction and Lectin Affinity Chromatography:** Pectoral muscle explants dissected from 50–100 11–12-d chick embryos or skeletal muscle fibroblasts grown in roller bottles were extracted with Nonidet P-40 detergent using previously described procedures (35), except that the  $\text{Ca}^{++}$  concentration was 1 mM in all buffers. The muscle extract was fractionated by wheat germ agglutinin (WGA) affinity chromatography using described procedures (35). When the various fractions were exposed to cells in an antiserum-blocking assay, the detergent was removed using Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, CA) as described previously (35).

## RESULTS

We used five known inhibitors of myotube formation to help elucidate the nature of the molecules responsible for the specific,  $\text{Ca}^{++}$ -dependent adhesion of myoblasts. Initially, we had to determine the conditions required to block fusion of EGTA-blocked cultures (initiated by addition of  $\text{Ca}^{++}$ ) since these were the myoblasts to be assayed for their ability to aggregate in suspension. Therefore, we tested various concentrations and addition times for tunicamycin, sodium butyrate, chloroquine, TFP, and 1,10 phenanthroline. Consistent with the observations of other investigators, all five agents inhibited

myotube formation (data not shown). The following concentrations and times of addition (hours after plating the cells) were found to be the optimal conditions (i.e., the lowest effective concentration and shortest incubation time) for blocking myotube formation initiated by adding 1–2 mM  $\text{Ca}^{++}$  to 50–52 h EGTA-blocked myoblasts: chloroquine, 10  $\mu\text{M}$ , 5 h; TFP, 2  $\mu\text{M}$ , 24 h; sodium butyrate, 2 mM, 24 h; tunicamycin, 0.1  $\mu\text{g/ml}$ , 31–34 h; 1,10 phenanthroline, 10  $\mu\text{g/ml}$ , 48 h. Unless otherwise indicated these conditions were then used to assess the effect of the myotube inhibitors on myoblast aggregation.

For aggregation assays, the cells were grown in the presence of EGTA and the myotube inhibitors were added at the concentrations and times described above. Approximately 51 h after plating, the myoblasts to be used in the aggregation assays were detached from the substratum using the adhesion-perturbing antiserum described in Materials and Methods and assayed for their ability to aggregate in both the absence and presence of  $\text{Ca}^{++}$  as described in Materials and Methods. The antiserum used to harvest the myoblasts neither aggregated the cells in the absence of  $\text{Ca}^{++}$  during the 20-min incubation nor inhibited their aggregation in the presence of  $\text{Ca}^{++}$ , even when added to the assay at a 10-fold higher concentration than that used to detach the myoblasts from their substratum (Table I). Thus, antibody-induced agglutination of cells does not occur under the conditions of the assay. Harvesting the cells using the antiserum resulted in an essentially pure myoblast population free of cell surface changes that might occur by detaching the cells with EDTA. Calculated from the results of 17 experiments, the average percentage of aggregated cells in the absence of  $\text{Ca}^{++}$  was 16% (range, 8–28%) and that in the presence of  $\text{Ca}^{++}$  was 55% (range, 41–69%). The variability from day to day is not entirely understood but is probably due to the condition of the cells, their precise concentration, and perhaps the expression of the molecules involved in cell-cell adhesion. In any case, the data are most easily understood and compared when they are expressed as a function of the  $\text{Ca}^{++}$ -plus control (i.e., percent aggregation in treatment divided by percent aggregation in control with  $\text{Ca}^{++}$  times 100).

Sodium butyrate, chloroquine, TFP, and 1,10 phenanthroline added under conditions that blocked myotube formation all had no effect on  $\text{Ca}^{++}$ -dependent myoblast aggregation (Table II and Fig. 1), implying that their effectiveness in inhibiting myotube formation is not due to a perturbation of the events involved in myoblast recognition and adhesion. In contrast, tunicamycin prevented the  $\text{Ca}^{++}$ -dependent adhesion of the myoblasts (Table II and Fig. 1), which suggests that this agent inhibits myotube formation by preventing  $\text{Ca}^{++}$ -dependent myoblast adhesion. For complete inhibition, the tunicamycin had to be added ~18 h before the aggregation assay. Addition 3–5 h before the aggregation assay resulted in only partial or no inhibition of myoblast aggregation. The effect of tunicamycin is not due to a general inhibition of protein synthesis during the 18–20-h incubation since TCA precipitation of cells metabolically radiolabeled with  $^3\text{H}$ -amino acids showed little effect on protein synthesis (Table III). On the other hand, treatment with cycloheximide, an inhibitor of both protein synthesis and  $\text{Ca}^{++}$ -dependent myoblast adhesion (33), during the same period showed a marked reduction in the incorporation of  $^3\text{H}$ -amino acids into TCA-precipitable counts (Table III). Although tunicamycin prevents myoblast aggregation, its effect can be inhibited by simultaneous addition of the protease inhibitor leupeptin

TABLE I. Myoblast Aggregation in the Presence of the Cell-Substratum Adhesion-perturbing Antiserum

Conditions of assay	% $\text{Ca}^{++}$ -plus control
1 mM $\text{Ca}^{++}$	100
No $\text{Ca}^{++}$	24
No $\text{Ca}^{++}$ , antiserum	27
1 mM $\text{Ca}^{++}$ , antiserum	96

51-h EGTA-blocked, fusion-competent myoblasts were harvested with the cell-substratum adhesion-perturbing antiserum (1:100 dilution) as described in Materials and Methods. The cells were aggregated at a concentration of  $2.5 \times 10^5$  cells/ml also as described there. The antiserum was added to the aggregation assay at a dilution of 1:20. The results are expressed as a percentage of aggregation in the  $\text{Ca}^{++}$ -plus control and represent the average of two experiments.

(Table IV), suggesting that the carbohydrate residues protect the glycoproteins from proteolytic degradation.

To determine whether or not the inhibitors that have no effect on myoblast aggregation block fusion of myoblasts after the cells have been aggregated in the presence of  $\text{Ca}^{++}$ , the myoblast clusters formed at a cell concentration of  $1 \times 10^6$  cells/ml were incubated overnight at 37°C in the continued presence of the inhibitors. In the control, cells aggregated in the presence of  $\text{Ca}^{++}$  (and absence of inhibitors) and incubated overnight in  $\text{Ca}^{++}$  fused into large, multinucleate myoballs (Fig. 2A) that could not be disrupted by treatment with 1 mM EDTA plus 0.1% trypsin. If we omitted  $\text{Ca}^{++}$  from the medium during the overnight incubation of aggregates formed in the presence of  $\text{Ca}^{++}$ , no fusion occurred (Fig. 2B) and the cells could be dispersed by trypsin. Similarly, cells treated with sodium butyrate, chloroquine, TFP, or 1,10 phenanthroline and aggregated in the presence of  $\text{Ca}^{++}$  failed to fuse even though  $\text{Ca}^{++}$  was present during the overnight incubation (Fig. 2, C, E, F, and G). In contrast to the above inhibitors of myotube formation, tunicamycin did not appear to prevent the fusion of cells in small aggregates that formed during the aggregation assay or during the overnight incubation (Fig. 2D).

Because  $\text{Ca}^{++}$  plays such an important role in myogenesis, we further investigated its relationship to the molecules implicated in myoblast adhesion. Knudsen and Horwitz (33) reported that trypsin treatment in the absence of  $\text{Ca}^{++}$  of fusion-competent myoblasts destroys their ability to aggregate when  $\text{Ca}^{++}$  is restored. Here we note that if 1–10 mM  $\text{Ca}^{++}$  is added to the cells 5 min before their treatment with trypsin, the effect of the enzyme is greatly diminished (Table V). This result suggests that a  $\text{Ca}^{++}$ -initiated conformation may protect the adhesion molecules from the action of trypsin.

Following a now well established paradigm for studying adhesive events in cells (reviewed in reference 11), we raised an antiserum against fusion-competent myoblasts in order to generate antibodies that bind to the adhesion molecules, perturb cell-cell adhesion, and, therefore, act as probes for the presence of adhesion-related molecules. We generated such an antiserum by injecting a rabbit with fusion-competent myoblasts harvested as described in Materials and Methods. The antiserum raised against myoblasts (anti-myoblast) blocks both  $\text{Ca}^{++}$ -dependent myoblast aggregation and myotube formation (Table VI). The effect of the antiserum on myoblast aggregation can be blocked by a Nonidet P-40 extract of chick pectoral muscle but not by an extract of cultured chick fibroblasts containing a similar amount of protein (Table VI). Preliminary experiments designed to purify the antiserum-blocking activity revealed that the blocking activity was en-

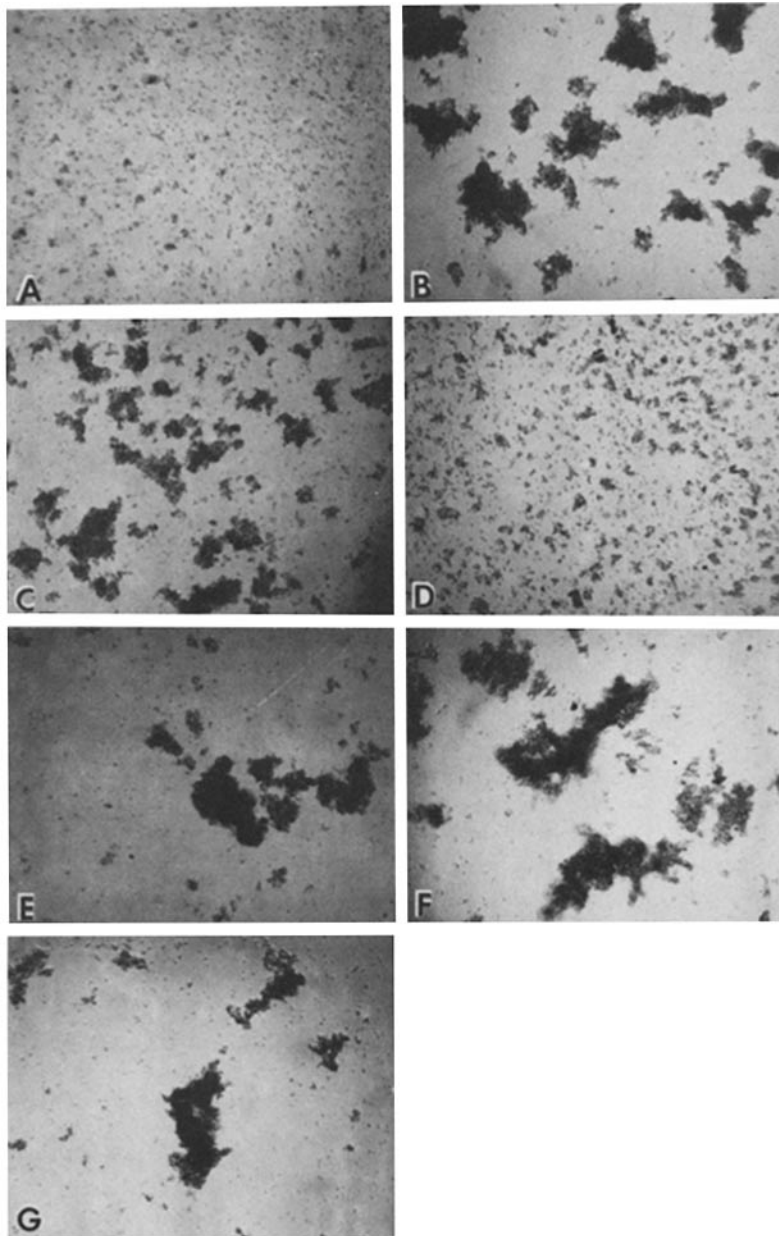


FIGURE 1 Myoblast aggregation in cultures treated with known inhibitors of myotube formation. EGTA-blocked myoblasts were harvested 51 h after plating and mixed at a concentration of  $1 \times 10^6$  cells/ml in the absence (A) or presence (B) of 1 mM  $Ca^{++}$  at  $37^\circ C$  for 20 min. The following inhibitors were added to the cultures at concentrations and times indicated in the legend to Table II: C, sodium butyrate; D, tunicamycin; E, 1,10 phenanthroline; F, TFP, and G, chloroquine.  $\times 40$ .

TABLE II. Quantitation of  $Ca^{++}$ -dependent Myoblast Aggregation in Cultures Treated with Known Inhibitors of Myotube Formation

Treatment of cells	% $Ca^{++}$ -plus control
1 mM $Ca^{++}$	100
No $Ca^{++}$	29
Chloroquine, 1 mM $Ca^{++}$	87
TFP, 1 mM $Ca^{++}$	93
1,10 Phenanthroline, 1 mM $Ca^{++}$	93
Sodium butyrate, 1 mM $Ca^{++}$	100
Tunicamycin, 1 mM $Ca^{++}$	18

EGTA-blocked myoblasts were harvested 51 h after plating as described in Materials and Methods and mixed at a concentration of  $2.5 \times 10^5$  cells/ml for 20 min at  $37^\circ C$ . Inhibitors were added at the following concentrations and hours after plating: chloroquine, 10  $\mu M$  at 5 h; TFP, 2  $\mu M$  at 24 h; tunicamycin, 0.1  $\mu g/ml$  at 31-34 h; 1,10 phenanthroline, 10  $\mu g/ml$  at 48 h. The data represent the average of at least three experiments and are expressed as a function of percent aggregation in the presence of  $Ca^{++}$ . In the absence of  $Ca^{++}$ , the percent aggregation in the presence of the inhibitors was similar to that of the  $Ca^{++}$ -minus control.

riched  $\sim 10$ -fold by lectin affinity chromatography using immobilized wheat germ agglutinin. This result is consistent with the hypothesis that the molecules that mediate  $Ca^{++}$ -dependent myoblast adhesion are cell surface glycoproteins. Further purification of these glycoproteins is in progress.

## DISCUSSION

Over the past years, investigators studying myogenesis have reported a number of agents and cell culture conditions that inhibit the in vitro formation of multinucleate muscle cells. These inhibitor studies have helped to define the events leading to myotube formation and to generally implicate calcium (49), proteins (33), glycoproteins (18, 43), lipids (25), and the cytoskeleton (24) as molecules that have roles in the construction of multinucleate muscle cells. The complexity of the process of myotube formation has made it difficult to identify the molecules involved and to understand when in

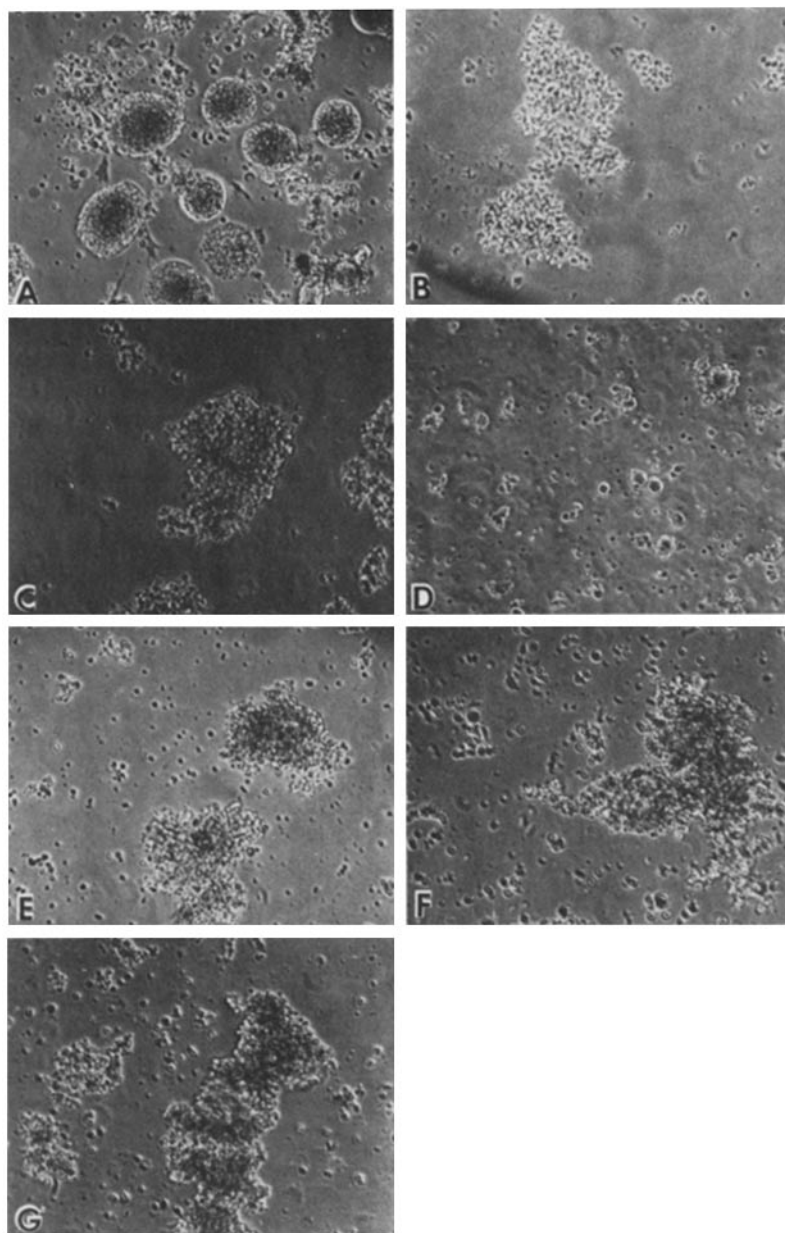


FIGURE 2 Myoball formation. EGTA-blocked myoblasts were harvested 51 h after plating and mixed at a concentration of  $1 \times 10^6$  cells/ml in the presence of  $\text{Ca}^{++}$  and the inhibitors that had been added to the cultures at the concentrations and times indicated in the legend to Table II. The aggregates were then incubated overnight in the continued presence of the inhibitors and/or 1 mM  $\text{Ca}^{++}$ , except in *B* where  $\text{Ca}^{++}$  was omitted. *A*, no inhibitor; *B*, no inhibitor (no  $\text{Ca}^{++}$ ); *C*, sodium butyrate; *D*, tunicamycin; *E*, 1,10 phenanthroline; *F*, TFP; and *G*, chloroquine.  $\times 40$ .

TABLE III. Metabolically Incorporated TCA-precipitable Radioactivity in Tunicamycin-treated Cells

Treatment of cells	cpm	% Control
Control	3,786 (7,022)	100 (100)
Tunicamycin (0.1 $\mu\text{g/ml}$ )	3,351 (6,447)	88 (92)
Cycloheximide (5 $\mu\text{g/ml}$ )	1,056 (2,157)	28 (31)

Reagents were added to EGTA-blocked cultures 31–32 h after plating and  $^3\text{H}$ -amino acid incorporation was assayed as described in Materials and Methods. Numbers in parentheses express the results of a duplicate experiment.

the process of myogenesis they are synthesized and used. The mechanism by which calcium promotes myoblast fusion is unknown, as is the exact function of the cytoskeleton. By focusing on the myoblast recognition/adhesion step of myogenesis, it is possible to refine the existing molecular information provided by reagents known to inhibit myotube formation and to begin to understand myogenesis at a molecular level.

TABLE IV. Effect of the Protease Inhibitor Leupeptin on Tunicamycin Inhibition of Myoblast Aggregation

Conditions of assay	% $\text{Ca}^{++}$ -plus control
1 mM $\text{Ca}^{++}$	100
No $\text{Ca}^{++}$	40
Tunicamycin, no $\text{Ca}^{++}$	27
Tunicamycin, 1 mM $\text{Ca}^{++}$	50
Leupeptin, no $\text{Ca}^{++}$	61
Leupeptin, 1 mM $\text{Ca}^{++}$	91
Tunicamycin, leupeptin, no $\text{Ca}^{++}$	47
Tunicamycin, leupeptin, 1 mM $\text{Ca}^{++}$	105

Tunicamycin (0.075  $\mu\text{g/ml}$ ) and leupeptin (50  $\mu\text{M}$ ) were added 31–32 h after plating and were present during the aggregation assay. 51 h after plating the myoblasts were harvested and aggregated under the above conditions as described in Materials and Methods. At higher concentrations of tunicamycin the leupeptin was less effective at protecting against the effect of tunicamycin on myoblast aggregation. The high percentage of aggregation in the presence of leupeptin and absence of  $\text{Ca}^{++}$  was observed repeatedly and may mean that leupeptin protects  $\text{Ca}^{++}$ -independent aggregation against endogenous protease activity.

TABLE V. Effect of Trypsin Treatment in the Absence or Presence of  $Ca^{++}$  on  $Ca^{++}$ -dependent Myoblast Aggregation

Treatment	% $Ca^{++}$ -plus control
1 mM $Ca^{++}$	100
0.01% trypsin, 1 mM EGTA	2
0.01% trypsin, 1 mM $Ca^{++}$	62
0.01% trypsin, 10 mM $Ca^{++}$	88

Cells were harvested as described in Materials and Methods and incubated for 5 min at room temperature in the presence of  $Ca^{++}$  or EGTA before incubation with trypsin at 37°C for 10 min. The reaction was stopped with excess soybean trypsin inhibitor, and the cells were aggregated in the presence of  $Ca^{++}$  as described in Materials and Methods.

TABLE VI. Effect of the Antiserum Raised against Fusion-competent Myoblasts on  $Ca^{++}$ -dependent Myoblast Aggregation and Myotube Formation

Conditions	% $Ca^{++}$ -plus control	
	Aggregation	Myotube formation
$Ca^{++}$	100	100
No $Ca^{++}$	26	9
$Ca^{++}$ , anti-myo	47	30
$Ca^{++}$ , anti-myo, muscle extract	93	—
$Ca^{++}$ , anti-myo, fibroblast extract	52	—
$Ca^{++}$ , anti-myo, WGA-minus material	42	—
$Ca^{++}$ , anti-myo, WGA-plus material	92	—

For the aggregation assays, EGTA-blocked myoblasts were harvested ~51 h after plating and mixed in suspension as described in Materials and Methods. The  $Ca^{++}$  concentration in all cases was 1 mM. The antiserum dilution varied between 1:30 and 1:40. The protein concentration of muscle and fibroblast extract and material not bound to WGA, prepared as described in Materials and Methods, was 2–4 mg/ml, and that of the material that bound to WGA was 0.2–0.4 mg/ml. Myotube formation was measured 20 h after the addition of  $Ca^{++}$  and a 1:20 dilution of anti-myo to 48-h EGTA-blocked cultures. The extent of both aggregation and fusion are expressed as a function of that observed in the presence of  $Ca^{++}$ . The aggregation data represent the average of 2–10 experiments, and myotube formation data are from a single experiment. Preimmune serum had no effect on myoblast aggregation or myotube formation.

Of the five inhibitors of myotube formation tested, only tunicamycin blocks the specific,  $Ca^{++}$ -dependent aggregation of fusion-competent myoblasts. This occurs at concentrations that have no effect on overall protein synthesis, even though to block myoblast aggregation totally the inhibitor has to be added ~18 h before the assay. Since addition of tunicamycin 1–5 h before the assay results in only a slight inhibition, we postulate that the adhesion glycoproteins are being synthesized ~34 h after plating the cells and that their expression is maximal a few hours before the EGTA-blocked myoblasts as a population display an optimal ability to adhere and fuse (i.e., 52 h after plating in low  $Ca^{++}$ ).

The studies reported here indicate that the myotube inhibitors sodium butyrate, chloroquine, TFP, and 1,10 phenanthroline do not exert their effect by preventing the specific adhesion of fusion-competent myoblasts. This conclusion is based on the observation that the inhibitors, when added to cultures at times and concentrations known to block myotube formation, do not block the  $Ca^{++}$ -dependent aggregation of myoblasts in suspension. These results suggest, for example, that neither metalloendoproteases nor calmodulin-binding proteins are involved in  $Ca^{++}$ -dependent myoblast adhesion. Rather, all four inhibitors block the fusion of the myoblasts after their adhesion in the presence of  $Ca^{++}$ . The mechanisms of action of these agents remain speculative and are best

expressed by the authors who first noted their ability to inhibit myotube formation (1, 9, 10, 15, 28).

Simultaneous addition of a protease inhibitor, leupeptin, blocks the effect of tunicamycin on  $Ca^{++}$ -dependent myoblast aggregation, which implies, as others have reported (44), that the carbohydrate residues do not participate in the adhesion event, but rather that they protect the glycoproteins from proteolytic degradation. This is consistent with the previous observation of Knudsen and Horwitz (33) that myoblast adhesion is not perturbed by treatment of cells with agents known to perturb carbohydrate residues such as galactose oxidase, mixed glycosidases, and neuraminidase.

Since myoblast aggregation is inhibited by treating cells with trypsin in the absence of  $Ca^{++}$ , it seems likely that the protein portion of the glycoprotein(s) is involved in the specific adhesion of the myoblasts. It is possible that the glycoprotein has a  $Ca^{++}$ -dependent conformation, since the presence of  $Ca^{++}$  appears to protect the adhesion molecules from degradation by trypsin. Such a  $Ca^{++}$ -dependent conformation has been reported for ovomorulin, the cell-cell adhesion molecule involved in early mouse embryogenesis (27).

Together with previous observations, the data presented here suggest that a distinct  $Ca^{++}$ -dependent recognition/adhesion event involving surface membrane glycoproteins is necessary, but not sufficient, for myoblast fusion. Molecules that mediate  $Ca^{++}$ -dependent adhesion have been implicated in interactions of lung cells (51), fibroblasts (56), neural retina cells (6, 20–22, 37, 53–55), liver cells (2, 5, 16, 41), teratocarcinoma cells (41, 52, 58, 59), epithelial cells (12), and in early embryogenesis (11, 14, 27, 47, 50). Myoblast adhesion differs from that in other cells, however, because it is accompanied by cell fusion. As Neff et al. (39) showed, membrane contiguity is apparent within 20–30 min after myoblast fusion is initiated by the addition of  $Ca^{++}$  to fusion-competent myoaggregates, which suggests that fusion closely follows adhesion. Indeed, it may be that  $Ca^{++}$ -dependent adhesion triggers a cascade of events that results in the formation of multinucleate muscle.

In summary, an essential step in myogenesis includes the specific interaction of myoblasts before their fusion. This event requires  $Ca^{++}$  and appears to be mediated by cell surface glycoproteins that may have a  $Ca^{++}$ -dependent conformation. After adhesion, the membranes between adjacent cells unite by a process that appears to require a particular lipid composition, the continued presence of  $Ca^{++}$ , and the cytoskeleton. These events may be initiated by  $Ca^{++}$ -dependent myoblast interaction and may result from a cascade of events triggered by surface glycoproteins that effect cell-cell adhesion.

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