

# Inhibition of Fusion of Embryonic Muscle Cells in Culture By Tunicamycin Is Prevented by Leupeptin

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**ABSTRACT** The carbohydrate requirement for alignment and fusion of embryonic quail muscle cells has been examined in tissue culture by use of tunicamycin (TM). The mononucleated, spindle-shaped proliferating myoblasts were treated with TM at various times before fusion and differentiation into multinucleated muscle fibers capable of spontaneous contraction.

Tm blocked protein glycosylation and expression of glycoproteins on the cell surface, and strongly inhibited fusion when added to cultures of differentiating muscle cells before the fusion "burst," but had no apparent effect on cell alignment. The inhibition of fusion was partially prevented when TM was administered in the presence of protease inhibitors such as leupeptin and pepstatin, but the inhibition of glycosylation was not prevented. Both glycosylation and fusion were completely restored to normal by the removal of the antibiotic from the medium.

These studies provide strong support for the idea that myoblast fusion is partially mediated by glycoproteins with asparagine-linked oligosaccharides. However, the requirement for the carbohydrate portion of the glycoprotein appears to be indirect in that it acts to stabilize the protein moiety against proteolytic degradation. Our findings do not rule out the possibility that oligosaccharide units of surface glycolipids have some role in myoblast fusion.

The cell surface is known to be greatly enriched in complex carbohydrates, primarily in the form of glycoprotein and glycolipids. There is considerable evidence to implicate cell surface carbohydrates in a variety of cellular reactions including surface recognition (20, 22, 23, 37), transport of metabolites across cell membranes (17, 26), synthesis of sulfated proteoglycans (33), and insertion and proper orientation of glycoproteins in the plasma membrane (13, 31, 32). There is also evidence that carbohydrates protect glycoproteins against proteolytic degradation (1, 3, 19, 24, 27, 34, 43, 44).

Since the discovery of high levels of developmentally regulated lectin activity in extracts of embryonic muscle tissue (for reviews, see references 2, 14, and 38), the involvement of surface carbohydrates in muscle fusion has been a matter of considerable interest. Although several published reports have presented data to support this assumption (9, 10), the involvement of cell surface carbohydrate and lectin in muscle fusion has not been established.

Muscle cell fusion may be conveniently studied in tissue culture. When explanted into tissue culture, the mononucleated, spindle-shaped, embryonic myoblasts proliferate for 3–4 d before they fuse and differentiate into multinucleated muscle fibers. 1–2 d later these fibers begin to contract spontaneously, and typical cross-striations can be observed (6, 15). If muscle fusion is mediated by the interaction between specific cell surface carbohydrates and cell surface carbohydrate-binding proteins (lectins), as has been suggested, then inhibition of glycosylation would be expected to block fusion.

We have used tunicamycin (TM) to investigate the requirement of protein glycosylation for fusion of embryonic quail breast muscle cells in culture. TM inhibits the synthesis of *N*-acetylglucosaminyl pyrophosphoryl polyisoprenol (11, 16, 39, 41, 42). Because this intermediate is required for the synthesis of *N*-glycoside-linked oligosaccharides, TM treatment results in synthesis of glycoproteins deficient in asparagine-linked oligosaccharides (4, 12, 19, 27, 28, 34, 39, 40).

We present evidence that myoblast fusion is blocked when protein glycosylation is strongly impaired by TM treatment. The protease inhibitors leupeptin and pepstatin substantially prevent the fusion block in TM-treated cultures without restoring glycosylation. These findings suggest that (a) a glycoprotein is required for fusion, but (b) the requirement for the carbohydrate portion is indirect in that it serves to stabilize the glycoprotein against proteolytic degradation, as previously reported (3, 19, 27, 43).

## MATERIALS AND METHODS

### Cell Culture

Myoblasts were isolated by mechanical dissociation from the pectoral muscle of 10-d-old Japanese quail and maintained in culture according to the modified procedure of Konigsberg (15) as described by Parent et al. (30). The cells were cultured in Eagle's Minimal Essential Medium (MEM) containing Earle's salts, 15% horse serum, 10% chick embryo extract, Eagle's nonessential amino acids, sodium bicarbonate (1.2 g/liter), 50 U/ml penicillin, 50 µg/ml streptomycin, and 50 µg/ml Fungizone. The cells were maintained in a humidified 37°C incubator with an atmosphere of 5% CO<sub>2</sub> and 95% air.

Primary cultures were seeded at a density of  $4 \times 10^6$  cells per 100-mm gelatin-coated Falcon tissue culture dish (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) and incubated for 24 h. Fibroblast-free secondary cultures were established by mild trypsinization and were seeded at a density of  $10^4$  cells per 60-mm gelatin-coated dish in 3 ml of growth medium. Fresh medium was added after 48 h and was not changed thereafter during the course of the experiment. The secondary cultures divided rapidly with a doubling time of ~10 h for 3 d. After a brief period of parallel alignment of cells (~8 h), a rapid fusion burst occurred with ~70% fusion in 24 h. The percent fusion increased to 85–95% during the next 2 d. Spontaneous twitching was observed after 5–6 d.

### Quantitation of Cell Fusion

Cultures were fixed in 2% glutaraldehyde, stained with Carazzi's hematoxylin and embedded in a thin layer of polyvinyl alcohol as described by Konigsberg (15). Improved accuracy of nuclei counting was achieved by treating the fixed cultures with ribonuclease (50 µg/ml) for 1 h at 37°C. The dried plastic film containing the cells was stripped from the plates and mounted onto glass slides. The number of nuclei in nine random fields (0.55 mm<sup>2</sup>) was counted by phase-contrast microscopy at a magnification of  $\times 125$ . The fraction of total nuclei in multinucleated fibers was calculated.

### Other Procedures

Protein synthesis and glycosylation were measured by the incorporation of [<sup>14</sup>C]leucine (2 µCi/ml) and [<sup>3</sup>H]mannose (5 µCi/ml), respectively, into TCA (10%) insoluble cellular material, protein was electrophoresed in SDS polyacrylamide gels, and fluorograms were prepared. Protein was determined as described previously (25, 27).

### Materials

TM was a gift from Dr. Gakuzo Tamura via the Drug Evaluation Branch of the National Cancer Institute. The radiochemicals L-[U-<sup>14</sup>C]leucine (sp act, 2 Ci/mmol), D-[U-<sup>14</sup>C]glucosamine (sp act, 238 mCi/mmol), D-[U-<sup>14</sup>C]galactose (200 mCi/mmol), L-[U-<sup>14</sup>C]fucose (250 mCi/mmol) and D-[2-<sup>3</sup>H]mannose (sp act, 2 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. Eagle's Minimum Essential Medium, horse serum, and antibiotic mixture were purchased from Grand Island Biological Co., Grand Island, N. Y. Collagen, trypsin, and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp., Freehold, N. J. Cycloheximide, leupeptin, pepstatin, and thiodigalactoside were purchased from Sigma Chemical Company, St. Louis, Mo.

## RESULTS

In the present study we have investigated the possible involvement of cell surface carbohydrates in the differentiation of embryonic muscle cells in culture. We explored this possibility by using TM to inhibit the glycosylation of the major class of surface glycoproteins, and evaluated its effect on the extent and rate of myoblast fusion to form myotubes.

## Effect of Tunicamycin on Protein Synthesis and Glycosylation

To examine the effects of TM on protein synthesis and glycosylation, quail myoblast cultures were incubated for 6 h in medium with and without TM; radiolabeled precursors were then added, and incubation continued for an additional 18 h. The results of a representative experiment are shown in Table I. The lowest concentration of TM (0.05 µg/ml) produced nearly maximal inhibition of mannose incorporation into total TCA-insoluble material (87–93%) and into specific glycoproteins (not shown) with minimal inhibition of leucine incorporation (12–16%). Higher concentrations of TM more strongly inhibited protein synthesis (35–41%) without further inhibiting glycosylation significantly. Similar results were obtained when glycosylation was monitored by the incorporation of glucosamine or fucose (not shown). However, galactose incorporation was less sensitive to inhibition by TM treatment, as expected, because galactose is also a major component of the carbohydrate structure of serine/threonine or hydroxylysine-linked oligosaccharide.

TM-treated cells remain viable for up to 72 h when incubated in the lowest concentration (0.05 µg/ml) of the drug. The cells appear healthy by phase-contrast microscopy and retain the capacity to synthesize protein and exclude trypan blue. In addition, the effects of TM on glycosylation and protein synthesis are reversible upon removal of the drug from the medium. At the highest concentration (0.2 µg/ml), or with longer exposures to the inhibitor, distinct cytotoxic effects are apparent, and the cells become highly vacuolated and come off the dish.

To study the relationship between the amount of mannose-labeled macromolecules on the cell surface and myoblast fusion, we labeled cells with [<sup>3</sup>H]mannose at various stages of development and estimated the surface fraction by sensitivity to trypsinization. For these studies, [<sup>3</sup>H]mannose was added to the cultures at consecutive 12-h intervals commencing 24 h after the establishment of the secondary culture. After labeling for 18 h the medium was withdrawn and the cultures were

TABLE I  
Effects of Tunicamycin on Incorporation of Leucine and Mannose into Total TCA-insoluble Fraction of Cultured Embryonic Muscle Cells

Precursor	Concentration of tunicamycin µg/ml	Incorporation into protein cpm/µg	Percent inhibition
L-Leucine U- <sup>14</sup> C	0	2,357 ± 169	0
	0.05	2,048 ± 142	14 ± 2
	0.10	1,806 ± 126	24 ± 3
	0.20	1,564 ± 83	38 ± 3
D-Mannose 2- <sup>3</sup> H	0	361 ± 47	0
	0.05	37 ± 11	90 ± 3
	0.10	31 ± 10	92 ± 3
	0.20	10 ± 3	98 ± 2

Cultured muscle cells were incubated with the radioactive precursor for 18 h at 37°C after a 6-h preincubation with or without TM, as described in Materials and Methods. Incorporation was determined by precipitation of protein with 10% TCA, solubilization in 0.5 N NaOH, dilution with Aquasol (New England Nuclear) of aliquots containing equal amounts of protein to a final volume of 20 ml. Radioactivity in these samples was measured by liquid scintillation spectrometry. The results represent the average of three experiments, and plus and minus represent the range.

incubated in phosphate-buffered saline (PBS, pH 7.4) containing 10  $\mu\text{g}/\text{ml}$  cycloheximide with or without 10  $\mu\text{g}/\text{ml}$  trypsin. After 10 min at 37°C, the incubations were terminated by the addition of soybean trypsin inhibitor to a final concentration of 15  $\mu\text{g}/\text{ml}$ . Both trypsinized and nontrypsinized cultures were extracted with 10% TCA, and the amount of radioactivity in macromolecular material was determined.

Approximately 85–90% of the incorporated radioactivity was released by trypsin treatment at all stages of differentiation. The total amount of radioactivity in both the intracellular and surface fractions increased by ~10-fold just before the “fusion burst” and declined slightly after fusion was completed.

The results indicate (a) that protein glycosylation is blocked by TM, (b) that trypsin-sensitive carbohydrates, presumably glycoproteins, are present on the surface of myoblast, and (c) that their synthesis and exposure on the cell surface are developmentally regulated.

### *Effect of TM on Myoblast Fusion*

The participation of surface glycoproteins in the fusion of undifferentiated myoblasts to form differentiated myotubes was examined by determining whether TM blocked muscle cell fusion. For these studies cells were incubated in culture medium with or without TM. The capacity of various concentrations of TM to inhibit myoblast fusion, when added at various stages of development, was tested. TM strongly inhibited the formation of myotubes at the lowest concentration without significantly impairing protein synthesis or cell growth. Both the number of nuclei in myotubes and the number of myotubes are substantially decreased in cells treated with TM. The effect is most prominent when TM is added 12–24 h before the onset of the fusion burst.

The lower percentage of fusion in the TM-treated cultures compared with that of the control could be a consequence of either the effects of the drug on protein synthesis, resulting in a lower cell density, or inhibition of glycosylation. To distinguish between these two possibilities, the cells were treated with various concentrations of cycloheximide to reduce protein synthesis to the level comparable to that of TM treatment. The results (not shown) indicated that the inhibition of fusion could not be accounted for by impairment in protein synthesis because fusion still occurred when protein synthesis was partially inhibited with cycloheximide.

To evaluate the effect of cell density on fusion, TM was added to proliferating myoblast cultures ~24 h before the onset of fusion and incubated for 72 h. The TM-treated cells continued to proliferate and a confluent monolayer was eventually obtained; however, fusion was almost completely inhibited (Fig. 1*b–d*). It is also apparent (Fig. 1*c* and *d*) that TM treatment does not inhibit or impair parallel alignment of cells. The effects of TM on glycosylation and fusion are simultaneously reversed when the drug is removed from the medium; in fact, >90% of the cells fuse within 36 h (Fig. 1*e*) and looked similar to fused control cultures (Fig. 1*f*).

These results suggest that an asparagine-linked, surface glycoprotein is required for myoblast fusion, and the inhibition of fusion by TM is not simply an indirect effect of decreased rate of growth or lower cell density.

### *Leupeptin Prevention of the TM Effect on Fusion*

Experiments were performed to determine whether the carbohydrate requirement was direct or indirect, because it had

been reported that nonglycosylated proteins are degraded more rapidly (3, 19, 27, 34–36, 43) and accumulate on the cell surface to a lesser extent than do the glycosylated protein species (7, 8, 25, 27, 43, 44). Based on these studies, it is plausible that inhibition of glycosylation also decreased the exposure or amount of the protein moiety on the cell surface because of enhanced proteolysis.

This possibility was investigated by supplementing the TM-treated cultures with leupeptin. Leupeptin is a relatively nontoxic inhibitor of proteases such as trypsin, papain, plasmin, and cathepsin B (18, 21). The cultures incubated with TM plus leupeptin do not show the inhibition of myoblast fusion seen with TM alone (Fig. 2*a*). The reduction in other specific surface glycoproteins by TM, as determined by  $\alpha$ -bungarotoxin ( $\alpha$ -BgT) binding to acetylcholine receptors (AChR) (Fig. 2*b*) is also partially prevented by leupeptin. Leupeptin has no effect on the inhibition of glycosylation by TM (Fig. 2*c*). The effect of leupeptin on myoblast fusion is potentiated by the simultaneous addition of pepstatin. The percent of cells with single nuclei in cultures treated with TM plus protease inhibitors is not significantly different from that obtained for control cultures; however, the individual myotubes contain fewer nuclei, and hence are smaller than in control cultures as shown in Fig. 3.

In the above experiment, TM and the protease inhibitors were added simultaneously to the proliferating myoblast cultures. Therefore, it is plausible that treatment with the protease inhibitors spared from degradation the preexisting, glycosylated protein required for fusion. This possibility was diminished by pretreatment of cells with TM for 24 h, before the addition of the protease inhibitors, to allow for depletion of the intracellular pool(s) and partial turnover of preexisting surface fraction. It was found that TM pretreatment did not significantly decrease the percent of fusion, which suggests that the glycosylated fusion protein(s) apparently did not exist in abundance 24 h before fusion.

These results indicated that the polypeptide portion of a glycoprotein(s) is sufficient to mediate membrane fusion. This suggests that the requirement for carbohydrate is indirect, in that it protects the protein component against cellular proteolysis.

## DISCUSSION

The biochemical mechanism(s) of muscle fusion is not known. It has been suggested that this complex process may involve, in part, a specific association between cell surface oligosaccharide receptors and specific cell surface carbohydrate-binding proteins (lectins). We have used TM to determine whether surface glycoproteins with asparagine-linked oligosaccharides are required for alignment and fusion of embryonic muscle cells in culture. We have found that treatment of myoblasts with TM resulted in three marked effects. First, protein glycosylation, as monitored by the incorporation of radiolabeled mannose into TCA-insoluble fraction, was almost completely abolished. Second, a comparatively large inhibition of myoblast fusion was observed, as measured by the fraction of total nuclei present in myotubes. Third, there was a marked decrease in the surface exposure of AChR as measured by  $\alpha$ -BgT binding.

These results suggest that protein glycosylation is required for muscle fusion. However, the carbohydrate requirement may be indirect, because it has been reported that nonglycosylated proteins degrade more rapidly (3, 19, 27, 34–36, 43) and accumulate on the cell surface to a lesser extent than do the

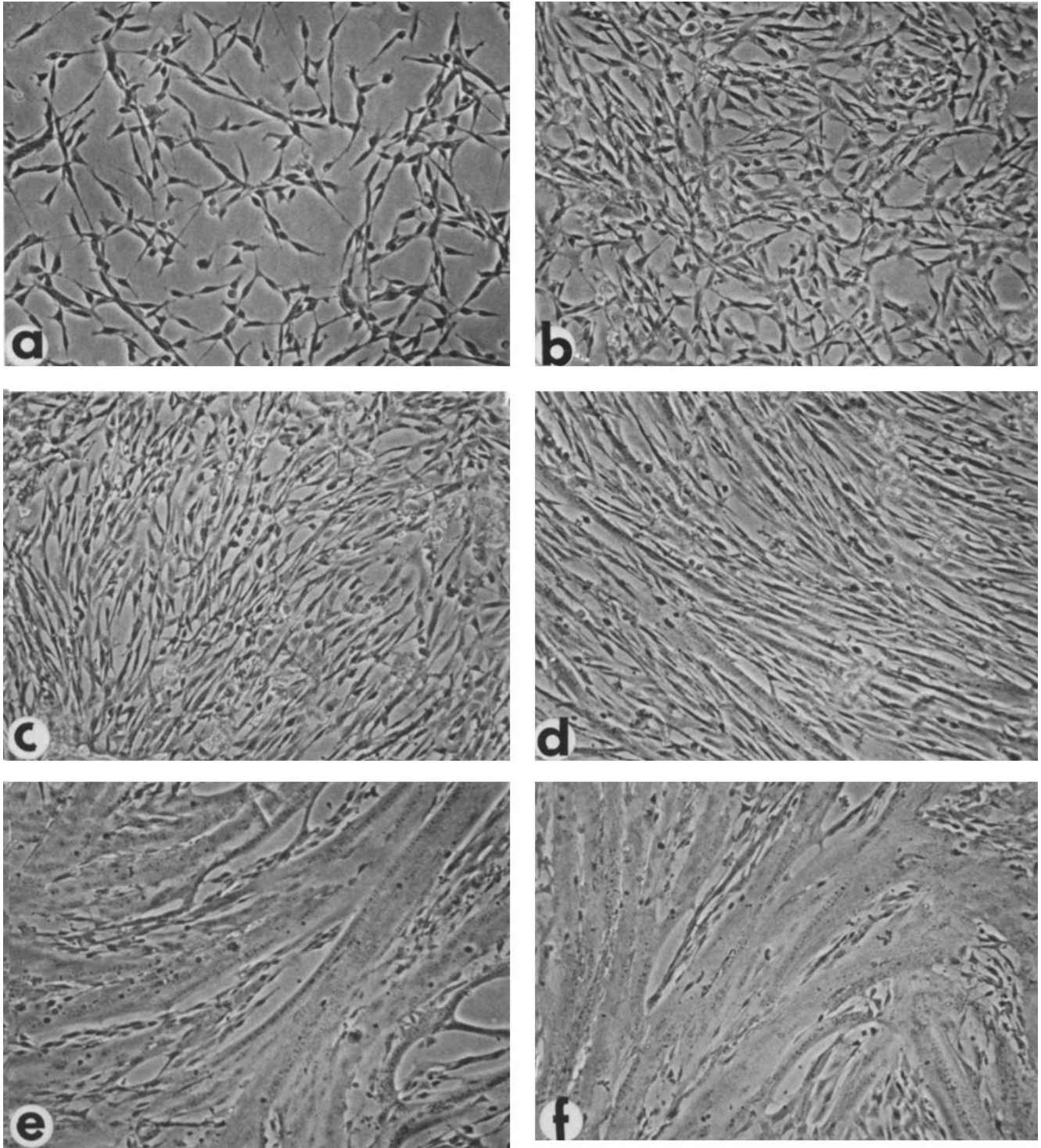


FIGURE 1 Reversal of the effect of TM on myoblast fusion. TM ( $0.05 \mu\text{g/ml}$ ) was added to cultures of embryonic muscle cells  $\sim 24$  h before the onset of fusion. Shown are phase-contrast photographs of myoblasts at the time of TM addition (a) and after incubation with the antibiotic for 24 (b), 48 (c), or 72 h (d). Reversal of inhibition 36 h after the 72-h pretreatment is shown in e, and a comparable untreated control is shown in f.

glycosylated protein species (7, 8, 25, 27, 43, 44). Based on these studies, it is plausible that inhibition of glycosylation in muscle cells also decreased the exposure or amount of the protein moiety in the cell surface because of enhanced proteolysis. We investigated this possibility by inhibiting proteolysis in TM-treated cells by use of the protease inhibitors. We found that the inhibition of fusion by TM in muscle cultures can largely be prevented with leupeptin and/or pepstatin, even

though glycosylation is still inhibited. We conclude that the asparagine-linked carbohydrate moieties on surface glycoproteins are not directly required for the fusion of embryonic muscle cells to form multinucleated myotubes. Rather, the protein component of glycoproteins with asparagine-linked oligosaccharides is required for muscle fusion, and we infer that the carbohydrate stabilizes the protein against proteolytic degradation.

The molecular mechanism of carbohydrate stabilization of glycoproteins to proteolysis is unknown. One mechanism may be that the carbohydrate moiety makes the glycoprotein inaccessible to the active site of proteolytic enzymes either through steric interference or a carbohydrate-induced conformational change in the protein. It has been reported that some isolated,

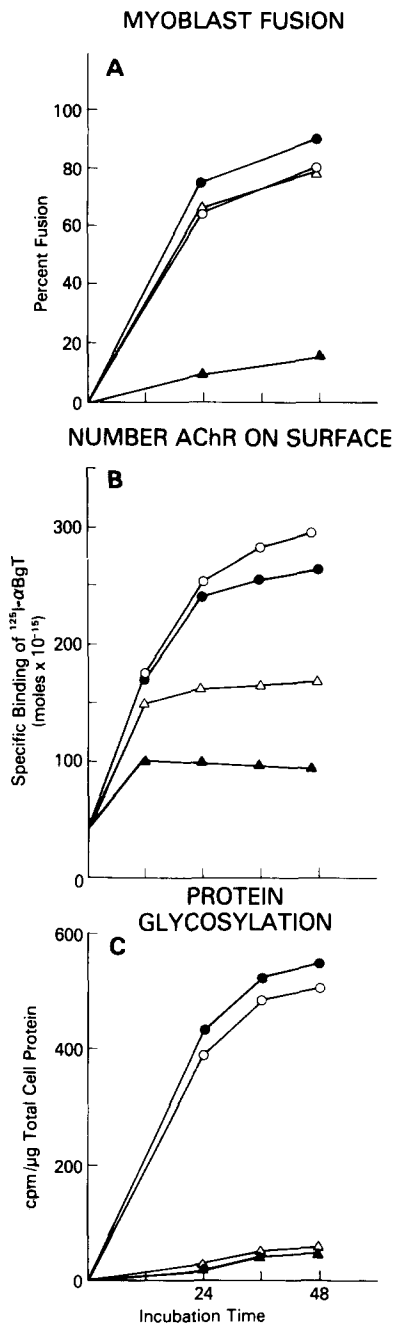


FIGURE 2 Effect of the simultaneous treatment with tunicamycin and leupeptin on fusion of myoblasts in culture. Muscle cells were treated with TM (▲; 0.05  $\mu\text{g}/\text{ml}$ ), leupeptin (○; 50  $\mu\text{M}$ ), or both (△) for 48 h. AChR on surface membranes of intact cells were measured by the specific binding of  $^{125}\text{I}$ - $\alpha\text{Bgt}$  as described by Prives and Olden (34). Cultures were incubated with  $^{125}\text{I}$ - $\alpha\text{Bgt}$  ( $10^{-8}$  M) in DMEM containing bovine serum albumin (1 mg/ml) for 1 h. Radioactivity that remained associated with the cells after five washes with 3-ml volumes of DMEM was counted by gamma spectroscopy. Specificity was established by the inhibition of  $^{125}\text{I}$ - $\alpha\text{Bgt}$  labeling in the presence of the competitive inhibitor decamethonium ( $10^{-5}$  M). Protein glycosylation and fusion were quantitated as described in Materials and Methods. ●, Control.

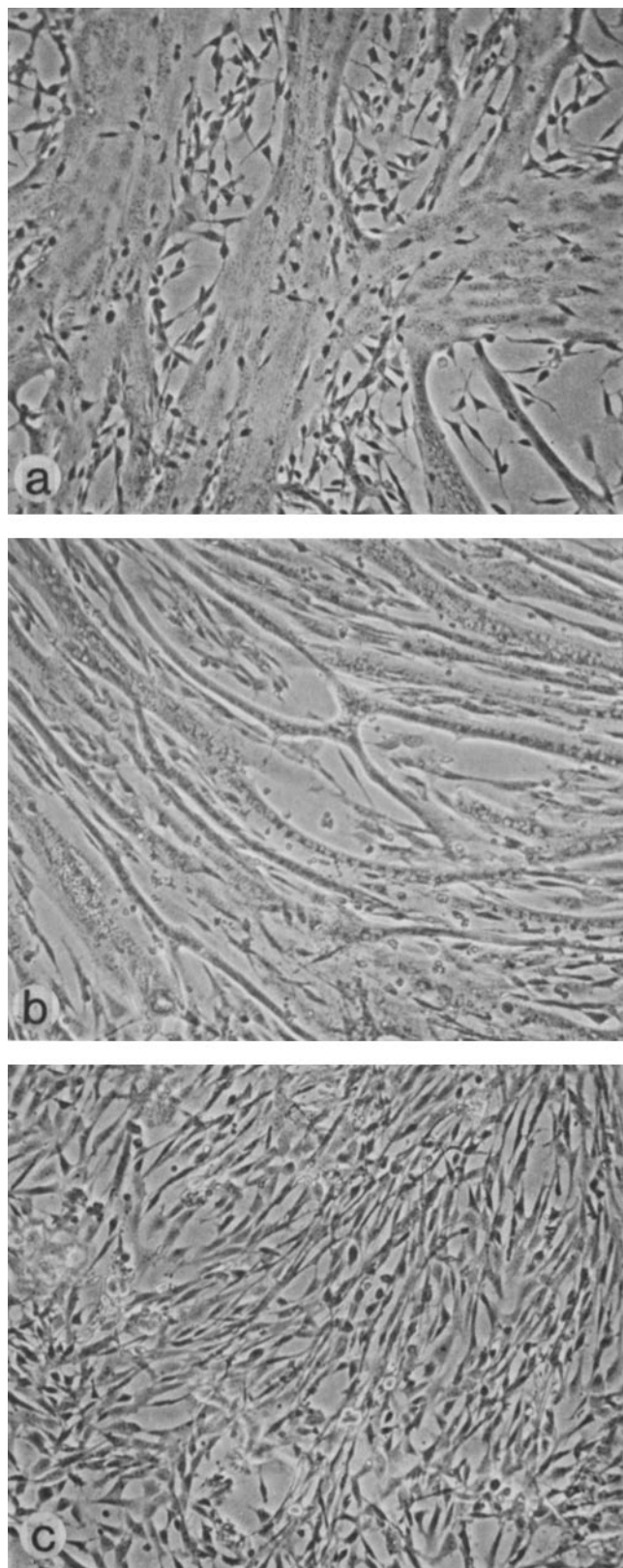


FIGURE 3 Phase-contrast micrographs of control cultures (a), and cultures treated with TM plus leupeptin (b), or TM alone (c), as described in the legend to Fig. 2.

nonglycosylated protein products are inherently more sensitive to proteases *in vitro* than the corresponding glycosylated species (3, 29). However, that exogenous lectin (concanavalin A) can decrease the rate of degradation of AChR in control

cultures of muscle cells, but not in cultures grown with TM (34), suggests an alternative mechanism of carbohydrate stabilization of glycoproteins. Endogenous lectins, which are found in abundance in muscle cells (2) and appear to be developmentally regulated (14), could serve to protect the glycoproteins against proteolysis during intracellular processing and transport. Impairment in processing of glycoproteins could occur because of an alteration in the carbohydrate binding (lectin) or in the carbohydrate moiety itself.

Another line of evidence that carbohydrates do not directly participate in myoblast fusion is the observation that myoblast fusion is not blocked by a variety of sugars. In fact, neither thiodigalactoside nor lactose, both potent inhibitors of muscle lectin agglutination of rabbit erythrocytes, had any effect on myoblast fusion (5).

The major conclusion from this work is that fusion of embryonic muscle cells is not mediated by the surface interaction between carbohydrate-binding proteins and asparagine-linked carbohydrate moieties that are both present on the cell surface. TM apparently inhibits fusion by decreasing the availability of a surface glycoprotein required for this process.

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