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Function of the Carbohydrate Moieties of Glycoproteins

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To determine the function of the carbohydrate moiety of glycoproteins, we have used tunicamycin, an analog of N-acetylglucosamine, to inhibit the glycosylation of N-glycosidically linked glycoproteins. First, we examined the effect of this drug on the intracellular processing, export and biological activity of fibronectin—the major cell surface glycoprotein of chick embryo fibroblasts. Chick fibroblasts treated with tunicamycin produced only nonglycosylated fibronectin and the export or secretion of the carbohydrate-free protein species was not totally impaired. We did observe that there was a substantial decrease in the absolute amount of nonglycosylated fibronectin on the cell surface and in the culture medium. This decrease was shown to be due to increased proteolytic degradation of the nonglycosylated protein species.

To examine the biological activity of nonglycosylated fibronectin, we compared the activities of the glycosylated and nonglycosylated forms of this protein utilizing in vitro assay procedures. We have shown that isolated, nonglycosylated fibronectin retained the biological properties characteristic of the glycosylated protein; they are: 1) promotion of cell-cell and cell-substratum adhesion, 2) restoration of normal behavior and phenotype to transformed cells, and 3) promotion of cell binding to collagen. The isolated, nonglycosylated protein was shown to be more sensitive to degradation by proteolytic enzymes, in agreement with the data obtained "in vivo."

The requirement of glycosylation for the export of acetylcholine receptor was also examined. We found that treatment of embryonic muscle cells in culture with tunicamycin did not inhibit the export of this protein to the cell surface. As with fibronectin, there was a substantial decrease in the amount of receptor present on the cell surface, due to enhanced proteolysis of the nonglycosylated protein. The simultaneous treatment of cells with the protease inhibitor leupeptin diminished the rate of degradation of the nonglycosylated receptor and restored the expression of receptor on the cell surface.

Finally, the requirement for N-glycosidically linked glycoproteins during differentiation of embryonic myoblasts into multinucleated, functional muscle fibers was also investigated. Tunicamycin blocked the expression of glycoproteins on the cell surface and strongly inhibited fusion when added to cultures of

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differentiating muscle cells prior to fusion. The inhibition of fusion was partially prevented when tunicamycin was administered in the presence of protease inhibitors such as leupeptin and pepstatin. Both glycosylation and fusion were completely restored to normal after removal of tunicamycin from the medium. These studies provide strong support for the idea that myoblast fusion is partially mediated by surface 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.

To elucidate the mechanism responsible for the enhancement of proteolysis of cell surface glycoproteins following treatment with tunicamycin, we investigated the effect of tunicamycin on the intracellular processing of proteolytic enzymes. Treatment of chick embryo fibroblasts with tunicamycin resulted in more than a 10-fold increase in the amount of protease activity released into the culture medium. The enzyme activity has been tentatively identified as cathepsin B based on substrate specificity, pH optimum and inhibition with leupeptin.

These results as well as extensive work by other investigators [see references [1-11] for recent reviews] suggest that the carbohydrate moiety of surface glycoproteins is not required for their synthesis, secretion or biological function, but instead helps to protect the protein against proteolytic degradation. In contrast, in agreement with the results of Neufeld et al [12-24] and Sly et al [15, 16], the carbohydrate moiety of lysosomal enzymes is required for their intracellular retention.

Key words: surface glycoproteins, myoblast fusion, glycosylation, proteolysis, cell adhesion, cathepsin B, intracellular processing, export/secretion, and tunicamycin

Glycoproteins are found in fungi, green plants, viruses, bacteria, and animal cells. Glycoproteins are common components of animal cell surfaces, and are also commonly present in lysosomes and among the products exported by the cell. Cell surface glycoproteins have been shown to play important roles in pinocytosis [17], differentiation [18-20], tumorgenesis [18, 21, 22], intercellular recognition and adhesion [21-28], as receptors for hormones and viruses [29], and as mediators of immunological specificity [30]. The secreted glycoproteins include hormones, immunoglobins and serum transfer factors [31-33]. Carbohydrate moieties of glycoproteins have also been implicated in the transport of metabolites across cell membranes [34, 35].

Although glycoproteins are present in great abundance in eukaryotic cells, the biological function of the constituent oligosaccharide units generally remains to be elucidated. The wide distribution and conserved structure of carbohydrate moieties of glycoproteins in animal tissues suggest their importance in an undefined, but universal, physiological process.

For the past four years, we have examined the role of glycosylation in the synthesis, processing, secretion, degradation, and biological activity of plasma membrane glycoproteins. For these studies, we have used the glucosamine-containing antibiotic called tunicamycin, an inhibitor of the synthesis of N-acetylglucosaminyl pyrophosphoryl polyisoprenol [36-39]. Since the formation of this intermediate is required for the synthesis of N-glycosidically linked oligosaccharides, treatment of cells with tunicamycin results in the synthesis of glycoproteins deficient in asparagine-linked oligosaccharides (see [24] and [27] for reviews). This drug was selected

because we and other workers had shown that the composition and chemical properties of the carbohydrates associated with fibronectin and acetylcholine receptor are characteristic of the "complex" type oligosaccharide structure linked by N-glycosidic bonds to asparagine [40–42]. There are an average of 4–6 such oligosaccharide side chains per monomer of fibronectin containing terminal galactose, sialic acid, and fucose residues [41, 42].

The acetylcholine receptor is a well characterized membrane glycoprotein of muscle. We used ^{125}I-α-bungarotoxin as a probe to study the effects of tunicamycin on the synthesis, degradation, and export of the acetylcholine receptor in embryonic muscle cultures [43,44]. The acetylcholine receptor of skeletal muscle is an integral membrane glycoprotein that is uniquely well characterized both pharmacologically and biochemically [see references 45 and 46 for recent reviews]. Direct analysis of the carbohydrate composition of purified acetylcholine receptor revealed the presence of mannose, galactose, and N-acetyl-D-glucosamine [reviewed in references 45, 46]. The total carbohydrate content is between 3% and 5%, but no specific function has been found for the carbohydrate component of acetylcholine receptor. Therefore, acetylcholine receptor may serve as a convenient model for detailed study of the function of the carbohydrate components of glycoproteins in the regulation of well-characterized plasma membrane properties. Acetylcholine receptor is of particular interest as its synthesis, distribution on the muscle cell surface, and degradation are tightly regulated during differentiation and upon innervation [45, 46].

Since the discovery of high levels of lactose-binding protein (lectin) activity in extracts of embryonic muscle cells [see references 47–49 for reviews], the involvement of surface carbohydrates in myoblast fusion has been vigorously investigated in several laboratories. Although several published reports have presented data interpreted to be supportive of such an involvement [50–53], the participation of cell surface carbohydrates and lectins in muscle fusion has not been established. The major argument against the involvement of the lactose-specific lectin in myoblast fusion is the finding that fusion is not inhibited by lactose nor its structural analogs [54]. In addition, most of the lectin activity appears to be localized in intracellular compartments [20].

Myoblast fusion can be conveniently studied in tissue culture since explanted mononucleated, spindle-shaped, embryonic myoblasts continue to proliferate for 3–4 days before they fuse and differentiate into multinucleated muscle fibers. If muscle fusion is mediated by the surface interaction of specific N-glycosidically linked carbohydrates with a carbohydrate-binding protein, then fusion should be blocked by treatment with tunicamycin.

We concluded from our studies that the asparagine-linked carbohydrate moieties are not required for the export or secretion of fibronectin, the acetylcholine receptor, or the myoblast fusion protein(s). In addition, the biological activities of fibronectin and the myoblast fusion protein(s) are retained by the nonglycosylated proteins. In contrast, the carbohydrate component is required for the intracellular retention of lysosomal hydrolases and for the protection of fibronectin, acetylcholine receptor, and the myoblast fusion protein(s) against proteolytic degradation.

METHODS

Cell Culture

Myoblasts were isolated by mechanical dissociation from the pectoral muscle of 10-day-old Japanese quail and maintained in culture according to the modified procedure of Konigsberg [55] as described by Parent et al [56]. The cells were cultured in Eagle's minimal essential medium (MEM) containing Earle's salts, 15% horse seum, 10% chick embryo extract, Eagle's nonessential amino acids, sodium bicarbonate (1.2 g/liter), 50 μ/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_2 and 95% air.

Primary cultures were seeded at a density of 4×10^6 cells/100 mm gelatin-coated Falcon tissue culture dish (Falcon Labware, Div. Becton, Dickson and Co, Oxnard, Calif) and incubated for 24 h. Fibroblast-free secondary cultures were established by mild trypsinization of primary cultures and reseeded at a density of 10^4 cells/60 mm gelatin-coated dish in 3 ml of growth medium. Fresh medium, added after 48 h, was not changed during the course of the experiment. The secondary cultures divided rapidly for three days with a doubling-time of approximately 10 h. After a brief period of parallel alignment of cells (approximately 8 h), a rapid fusion burst occurred with about 70% fusion in 24 h. The percent fusion increased to 85%–95% during the next two days, and spontaneous twitching was observed.

Primary chick embryo fibroblasts, prepared from ten-day-old White Leghorn embryos, were cultured in Eagle's Basal Medium supplemented with 10% tryptose phosphate broth, 5% heat-inactivated calf serum, 0.056% sodium bicarbonate, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine [57]. Cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. For protease secretion experiments, serum containing medium was aspirated, the cell culture was washed once with Eagle's Basal Medium containing 0.05% (W/V) polyvinylpyrolidone-40 and 1% penicillin-streptomycin, and incubated in this medium with or without tunicamycin (0.05 μg/ml).

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 [55]. 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 9 random fields (0.55 mm^2) was counted by phase contrast microscopy at a magnification of 125×. The fraction of total nuclei in multinucleated fibers was calculated.

Protease Assay

A. Cathepsin B. The protease activity released into the culture medium was determined by incubating the following reaction mixture at 37°C for 3 h: 50 μl of medium, 50 μl of 100 mM sodium acetate buffer (pH 4.4), and 5 μl (22.4 μM) of radiolabeled substrate (benzoyl-L-prolyl-L-phenylalanyl-L-arginyl-anilide, [aniline-^{14}C(U)] (sp act 10.7 mCi/mmole). The reaction, which is linear for at least 3 h, was terminated by heating the reaction mixture in a 100°C water bath for 2 min. One hundred μl of 200 mM borate buffer (pH 9.0) was added to neutralize the pH so

that the extraction of the radiolabeled aniline released by proteolysis would be maximized. The neutralized reaction mixture was extracted 3× with 250 μl of hexane, pooled, and counted in Econofluor (New England Nuclear). All determinations were done in triplicate.

B. Protease digestion of fibronectin. Protease digestions of glycosylated and nonglycosylated ^{14}C-leucine-labeled fibronectins were performed with 50 μg of each in 0.05 μg/ml pronase (Calbiochem) in 0.1 M sodium phosphate buffer (pH 7.4) at 37°C. Aliquots of 100 μl were removed at specified intervals, and radioactivity in soluble and acid-precipitable fractions was determined after precipitation with trichloroacetic acid.

Isolation of Fibronectin

Fibronectin was isolated from secondary cultures of chicken embryo fibroblasts by the urea extraction procedure described by Yamada et al [41, 58], and also by the immunoaffinity chromatography procedure as described by Olden et al [59]. The different methods of isolation did not alter the properties of the protein. ^{14}C-labeled fibronectin was prepared by incubation of chick embryo fibroblast cultures with 5 μCi/ml ^{14}C-leucine for 24 h prior to extraction.

Acetylcholine Receptor Assay

Acetylcholine receptor on surface membranes of intact muscle cells was measured by the specific binding of ^{125}I-α-bungarotoxin as described previously [43, 44, 60, 61]. The degradation of the ^{125}I-α-bungarotoxin- acetylcholine receptor complex was measured by the release of ^{125}I into the medium by cultures previously incubated with ^{125}I-α-bungarotoxin [43, 44, 60, 61].

Fibronectin Bioassay

Hemagglutination, effects on cell morphology, cell spreading, and cell attachment to collagen were assayed in the presence of glycosylated and nonglycosylated fibronectin as described previously [59, 62].

Other Procedures

Protein synthesis and glycosylation were measured by the incorporation of ^{14}C-L-leucine (2 μCi/ml) and ^{3}H-D-mannose (5 μCi/ml), respectively, into trichloroacetic acid (10%) -insoluble cellular material, protein was electrophoresed in sodium dodecylsulfate-polyacrylamide gels, and fluorograms were prepared as described previously [35, 40, 59]. Protein was determined according to the Lowry procedure [63], and amino sugar analyses were also performed as described elsewhere [59, 64].

Materials

Tunicamycin was a gift from Dr. Gakuzo Tamura via the Drug Evaluation Branch of the National Cancer Institute. The radiochemicals ^{14}C(U)-L-leucine (sp act 2 Ci/mmole), ^{14}C(U)-D-glucosamine (sp act 238 mCi/mmole), ^{14}C(U)-D-galactose (200 mCi/mmole), ^{14}C(U)-L-fucose (250 mCi/mmole), 2-^{3}H-D-mannose (sp act 2 Ci/mmole), ^{125}I-α-bungarotoxin (10–20 μCi/μg), and the radiolabeled protease substrate were purchased from New England Nuclear. Eagle's minimum essential medium, horse serum, and antibiotic mixture were purchased from Grand Island Biological Company. Collagen, trypsin, and soybean trypsin inhibitor were obtained

from Worthington Biochemical Corporation. Cycloheximide, leupeptin, pepstatin, and thiodigalactoside were purchased from Sigma Chemical Company.

RESULTS AND DISCUSSION

We shall describe the results and implications of recent experiments performed in our laboratory to determine the role of the carbohydrate moiety of glycoproteins. We have considered three possible functions for the N-glycosidically linked oligosaccharide components: 1) participation in cell-cell interactions, 2) involvement in mediation of biological activity, and 3) involvement in intracellular processing, segregation, and export.

Cell-Cell Interactions

Effect of tunicamycin on the surface morphology of chick embryo fibroblasts. The shape and surface morphology of chick embryo fibroblasts were examined by phase contrast and scanning electron microscopy. The control cells had the usual flattened, fibroblastic morphology at both 24 and 48 h with few apparent microvilli and blebs as shown in Figure 1. The morphology of tunicamycin treated cells was

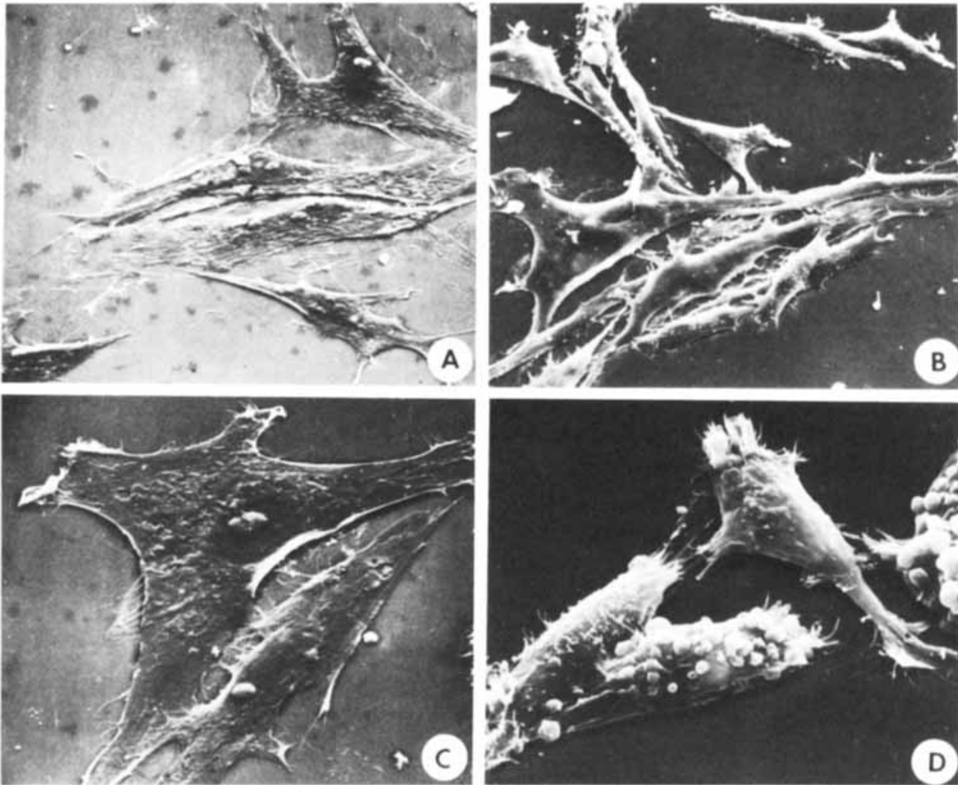


Fig. 1. Scanning electron microscopy of control and tunicamycin-treated cells. (A) Control chick embryo fibroblasts, 24 h × 720; (B) tunicamycin (0.05 μg/ml) 48 h × 720; (C) control chick embryo fibroblasts, 48 h × 1,000; (D) tunicamycin (0.2 μg/ml) 48 h × 2,000.

dramatically altered with a more rounded shape and exhibited numerous surface microvilli and blebs. The normal cell morphology was restored by the removal of tunicamycin from the growth medium. This change from flat to round shape is reminiscent of the changes that occur upon viral transformation of chick embryo fibroblasts. While the addition of exogenous fibronectin, isolated from normal chick embryo fibroblasts, restores transformed cells to a normal morphology [65, 66], it had only a minimal effect on the tunicamycin treated cells. It appears that the reduced amount of fibronectin cannot be completely responsible for the altered morphology in tunicamycin-treated cells. However, we do not know whether the tunicamycin treated cells have lost the capacity to bind fibronectin. For example, tunicamycin treated 3T3 lost the capacity to bind epidermal growth factor [67].

Effect of tunicamycin on myoblast fusion. The involvement of surface, N-glycosidically linked glycoproteins in the fusion of undifferentiated myoblasts to form differentiated myotubes was examined by determining whether tunicamycin blocked muscle cell fusion. For these studies, myoblasts were incubated in culture medium with or without tunicamycin. The capacity of various concentrations of tunicamycin to inhibit myoblast fusion, when added at various stages of development, is shown in Table I. Tunicamycin, at the lowest concentration, did not significantly impair protein synthesis or cell growth, but markedly inhibited the incorporation of ^{3}H-mannose into specific glycoproteins as shown in Figure 2. Similar results were obtained when glycosylation was monitored by the incorporation of glucosamine or fucose (Table II). However, galactose incorporation was less sensitive to inhibition by tunicamycin treatment (Table II), as expected, because galactose is also a major component of the carbohydrate structure of serine/threonine or hydroxylysine-linked oligosaccharide. When tunicamycin was added to proliferating myoblast cultures (Fig. 3a) approximately 24 h before the onset of fusion, the tunicamycin-treated cells continued to proliferate and a confluent monolayer was eventually obtained; however, fusion was almost completely inhibited (Fig. 3c) compared with untreated control culture (Fig. 3b). Both the number of nuclei per myotube as well as the number of myotubes are substantially decreased, and the effect is most prominent when tunicamycin is added 12–24 h before the onset of fusion

TABLE I. Effect of Tunicamycin on Myoblast Fusion

	Stage of development						
Concentration	Pre-fusion			Fusion		Post-fusion	
of TM (μg/ml)[a]	24 h	12 h	6 h	0	6 h	12 h	24 h
0	93% Fusion	93% Fusion	93% Fusion	93% Fusion	93% Fusion	93% Fusion	93% Fusion
0.05	7% Fusion	11% Fusion	35% Fusion	57% Fusion	78% Fusion	86% Fusion	93% Fusion
0.10	5% Fusion	10% Fusion	33% Fusion	52% Fusion	66% Fusion	81% Fusion	91% Fusion
0.20	2% Fusion	6% Fusion	20% Fusion	39% Fusion	59% Fusion	77% Fusion	86% Fusion

[a]The various concentrations of TM were added to the embryonic muscle cultures at the indicated stages of development. The amount of fusion in all cultures was determined at 48 h after the onset of fusion.

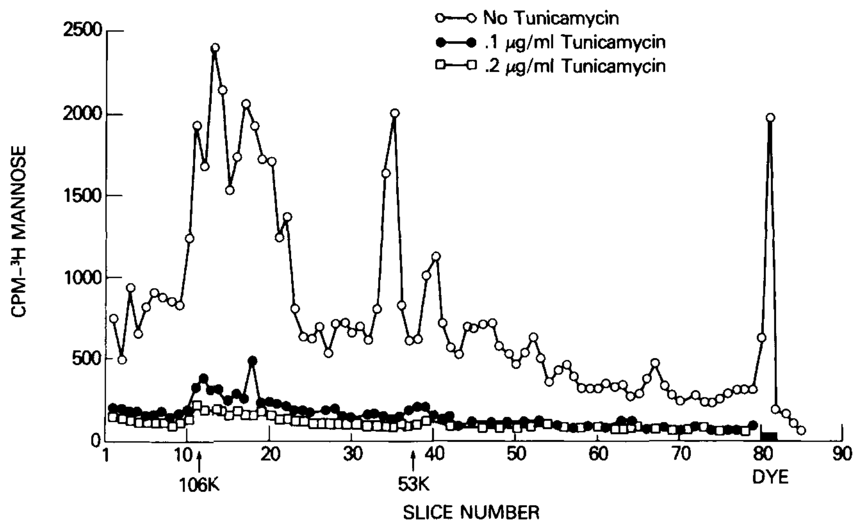


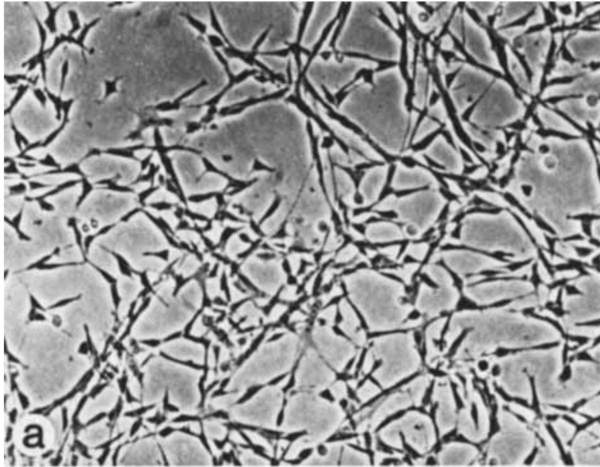
Fig. 2. Effect of tunicamycin on the incorporation of 2-^{3}H-D-mannose into myoblast glycoprotein. Following 6 h of preincubation in the presence or absence of 0.05 μg/ml tunicamycin, cells were further incubated for 18 h in the same media containing 2-^{3}H-D-mannose (5 μCi/ml). The cells were homogenized and electrophoresed in SDS-polyacrylamide gels (7.5%). The gels were sectioned into 1-mm slices, dissolved in 1.5 ml 30% hydrogen peroxide, diluted to 20 ml with Aquasol (New England Nuclear) and counted by liquid scintillation spectrophotometry.

TABLE II. Effects of Tunicamycin on Incorporation of Oligosaccharides Into Trichloroacetic Acid-Insoluble Fraction of Cultured Embryonic Muscle Cells

Precursor	Supplement	CPM/μg Protein	Percent inhibition
D-Mannose	−	296	0
2-^{3}H	+	23	92
D-Glucosamine	−	315	0
^{14}C (U)	+	11	97
L-Fucose	−	57	0
^{14}C (U)	+	3	95
D-Galactose	−	129	0
^{14} (U)	+	91	30

Cultured muscle cells were incubated with the radioactive precursors with (+) or without (−) TM, as described in legend to Figure 2. Shown is a representative experiment.

Fig. 3. Effect of tunicamycin on myoblast fusion. Tunicamycin (0.05 μg/ml) was added to cultures of embryonic muscle cells 24 h before the onset of fusion. Shown are phase-contrast micrographs of myoblasts at the time of tunicamycin addition (a) and after a 48 h incubation in absence (b) or presence (c) of the antibiotic.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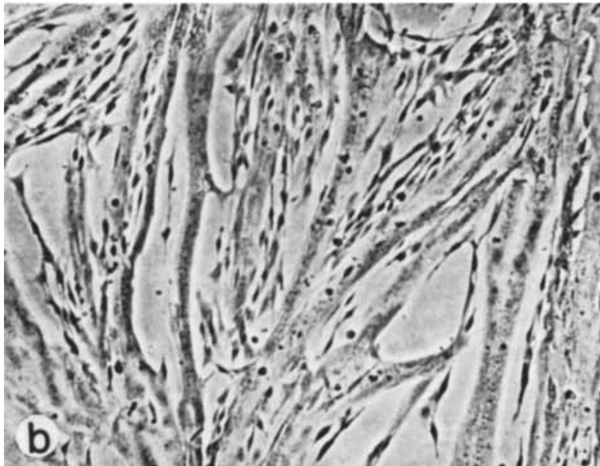
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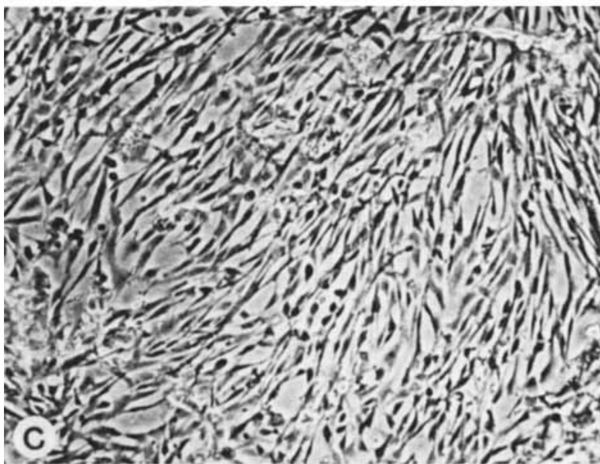
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(Table I). The effect of tunicamycin on glycosylation and fusion are simultaneously reversed when the drug is removed from the medium; in fact, greater than 90% of the cells fused within 36 h and looked similar to fused control cultures.

The lower percentage of fusion in the tunicamycin-treated cultures compared to the control could be a consequence of either the effects of the drug on protein synthesis, resulting in a lower cell density, or to 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 tunicamycin treatment. The results (not shown) indicated that the inhibition of fusion could not be accounted for by impairment in protein synthesis since fusion still occurred when protein synthesis was partially inhibited with cycloheximide.

These results suggest that an asparagine-linked, surface glycoprotein(s) is required for myoblast fusion.

Leupeptin reversal of the tunicamycin effect on fusion. Experiments were performed to determine whether the carbohydrate requirement was direct or indirect, since it had been reported that nonglycosylated proteins are degraded more rapidly [40, 44, 68–72] and accumulate on the cell surface to a lesser extent than the glycosylated protein species [40, 72]. 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 tunicamycin-treated cultures with leupeptin. Leupeptin is a relatively nontoxic inhibitor of proteases such as trypsin, papain, plasmin, and cathepsin B [73, 74] and has no effect on glycosylation [75]. The cultures incubated with tunicamycin plus leupeptin do not show the inhibition of myoblast fusion seen with tunicamycin alone (Fig. 4b and 4c, respectively). The effect of leupeptin on myoblast fusion is potentiated by the simultaneous addition of pepstatin (not shown). The percent of cells with single nuclei in cultures treated with tunicamycin plus protease inhibitors is not significantly different from that obtained for control cultures (Fig. 4a); however, the individual myotubes contain fewer nuclei and hence are smaller than in control cultures as shown in Figure 4.

In the above experiment, tunicamycin and the protease inhibitors were added simultaneously to the proliferating myoblast cultures. Therefore, it is plausible that treatment with the protease inhibitors spared the degradation of preexisting, glycosylated protein required for fusion. This possibility was diminished by pretreatment of cells with tunicamycin for 24 h, prior to the addition of the protease inhibitors, to allow for depletion of the intracellular pool(s) of glycosylated protein and partial turnover of the preexisting surface fraction. The 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 prior to fusion.

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

Biological Activity

To determine the role of the carbohydrate moiety in the mediation of the biological activity of fibronectin, we compared the activities of the glycosylated and nonglycosylated species of this glycoprotein using in vitro assay procedures. The carbohydrate-free protein was obtained by pretreating chick embryo fibroblasts with

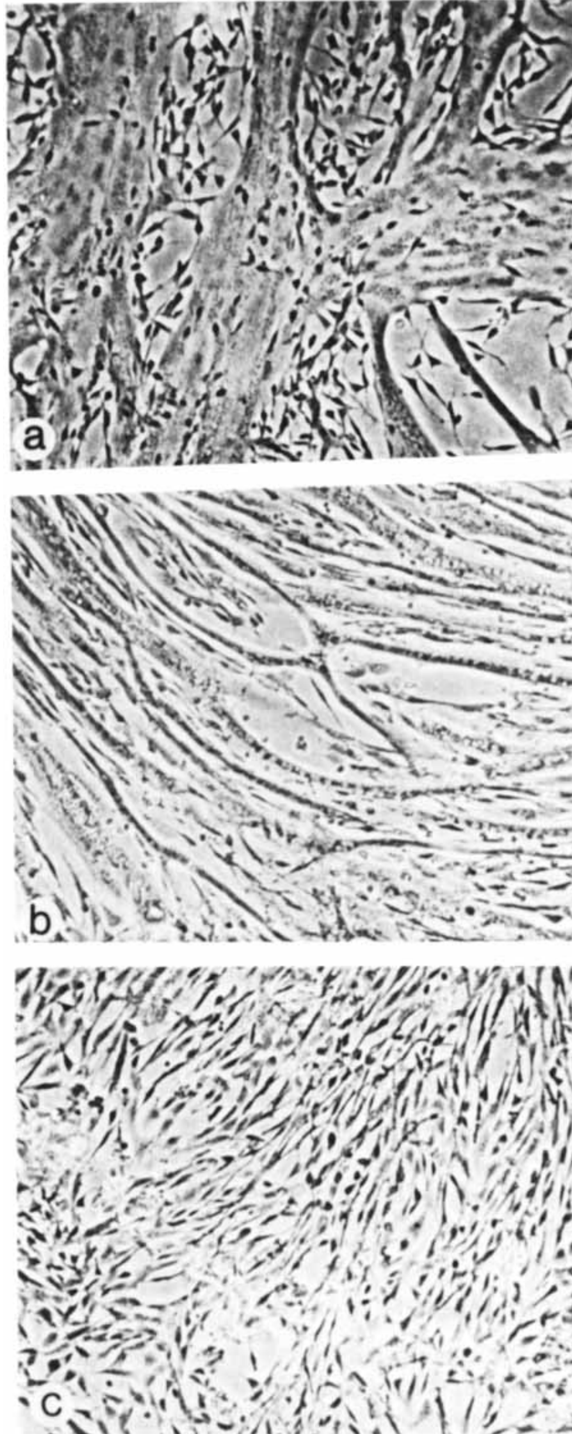

Fig. 4. Leupeptin reversal of the tunicamycin effect on fusion. Shown are phase-contrast micrographs of control cultures (a), and cultures treated with tunicamycin (0.05 μg/ml) plus leupeptin (50 μM) (b), or tunicamycin alone (c) for 48 h.

tunicamycin for 3.5 h to deplete the intracellular pool of glycosylated fibronectin. The pretreated cells were then trypsinized (0.25% trypsin for 10 min at 37°C) then replated and cultured in the presence of tunicamycin for another 24 h. The fibronectin synthesized during this 24 h interval was isolated by antibody-affinity chromatography as described [59].

The nonglycosylated fibronectin was 98% free of carbohydrate as determined by direct amino sugar analyses and decrease in incorporation of ^{14}C-glucosamine and ^{3}H-mannose compared to glycosylated fibronectin [59]. The absence of mannosyl and/or glucosyl sugar units was demonstrated by the inability of fibronectin, isolated from tunicamycin treated cells, to bind concanavalin A in contrast to the native protein [40, 59].

First, we measured the capacity of glycosylated and nonglycosylated fibronectin to agglutinate formalinized sheep erthrocytes. Secondly, we performed reconstitution experiments in which fibronectin was added to transformed cells (SV 40-3T3) deficient in the glycoprotein, and measured their capacity to restore the fibroblastic morphology characteristic of nontransformed cells. Finally, glycosylated and nonglycosylated fibronectin were compared with respect to their capacity to promote 1) the spreading of cells (Baby Hamster Kidney—BHK) on the surface of plastic tissue culture dishes, and 2) the binding of cells (Chinese Hamster Ovary—CHO) to collagen (type 1) coated dishes.

Each of these assays measures a slightly different feature of fibronectin's activity. Hemagglutination of formalin-fixed erythrocytes measures cell-cell adhesive interactions. For hemagglutination to occur, the protein must have several active sites which can interact with the surface of several cells resulting in intercellular linkage. The cell morphology assay measures the capacity of a protein to promote cell spreading and to alter the cell-cell interactions involved in parallel alignment of cells. The cell spreading assay probably requires that the adhesive molecule interacts with both the cell and the substratum. The collagen attachment assay is mediated by the interaction of fibronectin with the cell surface and also to the extracellular collagen matrix.

We found that nonglycosylated fibronectin was as effective as glycosylated fibronectin in 1) promoting cell spreading (half-maximal at a protein concentration of 1 μg/ml), 2) mediating ttachment of cells to collagen, 3) restoring normal fibroblastic phenotype, and 4) in agglutinating sheep erythrocytes. Therefore, we conclude that the carbohydrate moiety of fibronectin is not required for a variety of biological activities mediated by this glycoprotein. Similarly, the carbohydrate moiety is not required for the enzymatic activity of yeast invertase [68], the antiviral activity of interferon [76] nor for the infectivity of the vesicular stomatitis virus which is mediated by glycoprotein G [77].

EXPORT AND STABILIZATION

The biogenesis of plasma membrane, lysosomal and secretory proteins and their process of intracellular transport are thought to largely utilize the flow of intracellular membranes as envisaged by Palade [78]. According to this model these proteins are synthesized on polysomes attached to the rough endoplasmic reticulum, are segregated in the cisternal space of the rough endoplasmic reticulum, and are transported through the smooth surface elements of the rough endoplasmic

reticulum to the Golgi apparatus where the different classes of proteins are thought to become concentrated at special sites because they have been programmed for secretion sequestration in specific cellular organelles. The "differentiated" regions of the Golgi give rise to vesicles containing lysosomal, secretory and/or membrane proteins. These vesicles eventually transfer their content to the appropriate preexisting cellular organelle by membrane fusion or exocytosis.

The various compartments of the endoplasmic reticulum (ER)—Golgi membrane system are thought to be functionally connected by means of a shuttle wherein small vesicles bud off from one compartment and fuse with the next compartment. These vesicles apparently travel along well defined paths as they move through the various intracellular compartments of the endoplasmic reticulum-Golgi membrane system. Exactly how these membrane vesicles recognize the appropriate membrane partner remains enigmatic.

The selective assortment of lysosomal, secretory and membrane proteins must require a very sophisticated intracellular system. The recognition system is presumably composed of discrete molecular entities within the structural matrix of the ER-Golgi complex which recognize specific molecular structures on the exported products. The interacting components are probably complementary and associate in a noncovalent manner. Since most of the products processed by this system are glycoproteins, it is reasonable to speculate that the molecular recognition may occur by specific association between an oligosaccharide entity on the glycoprotein with a specific carbohydrate receptor. In fact, it has been proposed that the covalent attachment of carbohydrate to proteins is obligatory for the export or secretion of protein [31,79]. While most extracellular proteins do have carbohydrate as part of their molecular structures, several such proteins do not [31]; in addition, many glycoproteins are found inside the cell as lysosomal or membrane constituents.

Chick embryo fibroblast cultures treated with tunicamycin produce only nonglycosylated fibronectin and procollagen which are exported to the cell surface and secreted into the culture medium; however, the total amount of fibronectin on the cell surface was decreased by about 50% (not shown). Similarly, the amounts of fibronectin and procollagen released into the culture medium were also decreased as shown in Figure 5. By immunofluorescence analysis, we were able to obtain evidence for an altered intracellular distribution of fibronectin in the tunicamycin-treated cells. Fibronectin in tunicamycin-treated cells was found in large intracellular vesicles thought to represent expanded portions of the endoplasmic reticulum; whereas, fibronectin in control cells was diffusely distributed in granular perinuclear structures. However, the decrease in the amount of fibronectin on the cell surface and in the medium of tunicamycin-treated cultures is primarily due to enhanced proteolysis of the nonglycosylated protein and not to intracellular accumulation [40]. The enhanced proteolysis of nonglycosated fibronectin in vivo may be partially due to the increased proteolytic sensitivity of the protein component, since it is digested more rapidly by proteases in vitro [59, 80].

When embryonic muscle cells in culture were treated with tunicamycin, the expression of acetylcholine receptor on the surface was diminished to 10% of the number found in untreated cultures, and the nonglycosylated receptor turned-over at a rate 3–4 times faster than glycosylated receptor. The simultaneous treatment of cells with tunicamycin and leupeptin diminished the rate of nonglycosylated receptor degradation and partially restored the expression of receptor on the surface.

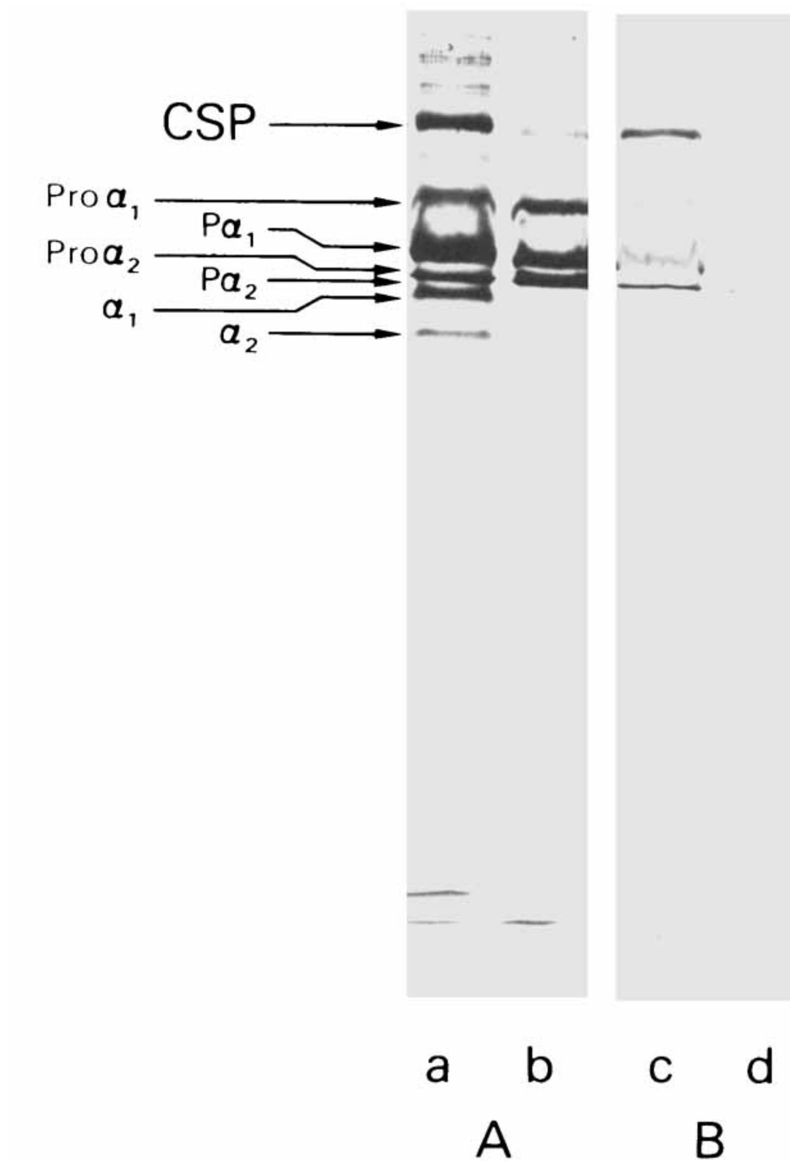

Fig. 5. Effect of tunicamycin on export of fibronectin and procollagen into the medium. Following 6 h of preincubation in the presence or absence of 0.05 μg/ml tunicamycin, cells were further incubated for 24 h in regular medium containing 2 μCi/ml L-proline (225 mCi/mmole, New England Nuclear), 50 mM ascorbate and 50 mM 3-aminoproionitrile. The medium was then centrifuged, precipitated with 10% TCA, homogenized in SDS, and electrophoresed. (A) coomassie blue-stained gels: medium of control cells (a), medium of treated cells (b). (B) autoradiograms: medium of control cells (c), medium of treated cells (d).

We concluded from these studies that the carbohydrate moieties are not required for the export or secretion of fibronectin, procollagen, or the acetylcholine receptor; however, glycosylation is necessary to protect fibronectin and acetylcholine receptor against proteolytic degradation [40, 44, 59].

Our studies that demonstrate that aberrant glycosylation can interfere with either the expression and/or stability of fibronectin and acetylcholine receptor may be generally applicable to other glycoproteins. For example, studies with a glycosylation-deficient mutant (AD6) of Balb/c 3T3 indicated that the extent of glycosylation affected the exposure of several plasma membrane-associated glycoproteins on the cell surface [81,82]. Recently, it has been reported that several Thy-1 negative mutants of lymphoma cells synthesize nonglycosylated or partially glycosylated T25 protein [72]. The amount of the T25 protein moiety synthesized is equivalent to wild-type cells; however, the nonglycosylated protein produced is not found on the cell surface nor is it shed into the culture medium in detectable amounts. Interestingly, the nonglycosylated protein in the mutant cells was degraded five to ten times faster than the wild-type glycosylated protein by intracellular proteases [72]. However, the decrease in exposure on the cell surface could not be entirely accounted for by enhanced proteolysis; hence, it was concluded that some intracellular accumulation must have occurred. Similarly, glycosylation is not required for the synthesis of the protein components of the ACTH and beta-LPH endorphin precursors in the neurointermediate lobe of the African frog (Xenopus laevis) [69,83], and the nonglycosylated precursors were rapidly degraded during intracellular transport; however, the nonglycosylated precursors that did escape degradation were processed and secreted normally [69, 83].

Studies with other systems suggest that the specificity of proteolytic cleavage during intracellular processing may be dependent on the presence of carbohydrate. For example, when the hemagglutinin precursor of HA_1 and HA_2 glycopeptides of influenza and fowl plaque virus is not glycosylated, it is rapidly degraded by intracellular proteases to abnormal cleavage products [70, 71]. Similarly, studies with Semliki Forest Virus have shown that the glycoprotein precursors (NSP63) of viral structural glycoproteins (E_2 and E_3) is not processed properly when glycosylation is inhibited by 2-deoxy-D-glucose or D-glucosamine [71, 84]. In this case, aberrant peptides are also produced in lieu of the normal structural protein products. The proteolytic processing of the nonglycosylated polypeptide precursor of the envelope protein of Rauscher murine leukemia virus is also impaired [85]. Perhaps the most compelling argument for the involvement of carbohydrate in protease digestion comes from studies with bovine pancreatic ribonucleases [86–88]. Glycosylated RNase B is less sensitive to tryptic digestion than native nonglycosylated RNase A [86]. Since these two proteins have identical amino acid sequences [87] and have similar, if not identical three-dimensional configurations [88], their differential sensitivity to trypsin is most likely due to the presence or absence of carbohydrate. This matter is currently being explored in our laboratory.

Other studies on the role of the carbohydrate moiety of glycoproteins are summarized in Table III. The requirement of carbohydrate for export or secretion of protein is not entirely resolved, although current evidence seems to indicate that it is not obligatory. Although some authors concluded that the export of nonglycosylated protein is impaired, this may not be the case. In all of these studies, some fraction of the nonglycosylated protein is, in fact, exported. Hence, further analyses

TABLE III. Effects of Inhibition of Glycosylation on Cell Surface and Secreted Glycoproteins

Glycosylated product	Inhibitor of glycosylation	Result	References
Cell surface proteins of Balb/c3T3 (iodinated)	Defect in N-acetyl-glusosamine synthesis	Absence or decreased labeling of many protein bands	Pouyssegur and Pastan [81, 82]
Cell surface proteins of BHK (iodinated)	2-Deoxyglucose	Decreased labeling of many bands	Hughes et al [84]
IgG of MOPC 21 cells	2-Deoxyglucose	80% inhibition of transfer into endoplasmic reticulum; 15% inhibition of secretion once transferred	Melchers [79]
IgG heavy chain of MPC-11 myeloma	Glycosylation mutation M 3.11	No alteration in kinetics of secretion	Weitzman and Scharff [89]
K-light chain K-46 of plasma-cytoma-46B	2-Deoxyglucose	60% inhibition of secre-tion, but same inhibition of total protein synthesis; 2-fold increase in intracellular pools	Eagon and Heath [90], Stark and Heath [91]
IgA of MOPC 315	Tunicamycin	85% inhibition of secre-tion; dilated endoplasmic reticulum containing IgA	Hickman et al [92]
IgE of IR 162 plasma cells	Tunicamycin	Complete inhibition of of secretion	Hickman et al [92]
Invertase and acid phophatase of yeast	Tunicamycin	Marked inhibition of secre-tion; no intracellular accumulation detected	Kuo and Lampen
Procollagen chicken embryo fibroblast	Tunicamycin	No inhibition of secretion; inhibition of proteolytic processing of COOH terminus	Duksin and Bornstein [94, 95] Olden et al [40]
Fibronectin of chick tendon fibroblasts and 3T3 cells	Tunicamycin	Inhibition of secretion into serum-free medium	Duksin and Bornstein [94, 95]
Fibronectin in chick embryo fibroblasts	Tunicamycin	No inhibition of secretion per se, enhanced proteolysis	Olden et al [40]
T25 glycoprotein of plasmacytoma	Genetic defect in glycosylation	Decrease in cell surface largely accounted for by enhanced proteolysis	Trowbridge et al [72]
Transferrin of rat liver	Tunicamycin	No inhibition of secretion	Struck et al [96]
Apoprotein B of chick liver	Tunicamycin	No inhibition of secretion	Struck et al [96]
ACTH-endorphin precursor	Tunicamycin	No effect on secretion per se	Loh and Gainer [69, 83]
Membrane glyco-proteins of BHK cells	Tunicamycin	No inhibition of export and shedding	Damsky et al [97]

(Continued next page)

TABLE III. (continued)

Glycosylated product	Inhibitor of glycosylation	Result	References
Acetycholine receptor in muscle cells	Tunicamycin	No inhibition of export enhancement of proteolysis	Prives and Olden, [44]
Viral glycoproteins E_2 E_3	2-deoxy-D-glucose and D-glucosamine	Impairs proteolytic processing	Schwarz et al [71] and Hughes et al [94]
Viral glycopeptides HA_1 HA_2	2-deoxy-D-glucose and D-glucosamine	Impairs proteolytic processing	Schwarz et al [70, 71]
Glycoprotein G of VSV	Genetic block in glycosylation	Impairs intracellular processing	Zilberstein et al [98]
Ovalbumin chick liver	Tunicamycin	No inhibition of secretion	Struck et al [96], Keller and Swank [99], and Coleman et al [100]
Colony-stimulating factor	Tunicamycin	No inhibiton of secretion or biological activity	Ayusawa et al [101]
Carboxypeptidase Y of yeast	Tunicamycin	No inhibition of secretion	Hasilik and Tanner [102]
Leukocyte Interferon	Tunicamycin	No inhibition of secretion or biological activity	Bose et al [76], Tamura and Sulkowski [103], and Fujisawa et al [104]
HLA antigen of lymphoblasts	Tumicamycin	No inhibiton of secretion	Nishikawa et al [105] and Plough et al [106]
Rhodopsin in retinal disk membranes	Tunicamycin	No inhibition of membrane insertion	Poncz and Kean [107]
Invertase and acid phosphatase in yeast	Tunicamycin	Inhibited secretion, entrapped in intracellular membranes	Onishi et al [108]
SFV membrane proteins	Tunicamycin	Inhibition of membrane insertion	Leavitt et al [109]

might indicate that the decreased amount of protein may not be due to inhibition of secretion per se, but to enhanced proteolysis, denaturation, or to reduced efficiency of intracellular segregation.

Processing of Lysosomal Enzymes

There is considerable evidence that the oligosaccharide moiety is required for the sequestration of hydrolases in lysosomes [see references 1–11, 110–112]. Sly and co-workers [111, 112] have advanced the model that delivery of hydrolytic enzymes to the lysosomes involves binding of newly synthesized enzymes bearing a man-6-PO_4 recognition marker to lectin localized in the endoplasmic reticulum. Membrane vesicles containing lectin-bound hydrolases bud from the GERL and either fuse with preexisting lysosomes or differentiate into lysosomes. One prediction of this model is that tunicamycin treatment of cells would block the current routing of newly syn-

thesized acid hydrolases to the lysosomes and may result in the secretion of acid hydrolases. To test this prediction, we incubated chick embryo fibroblast in medium containing tunicamycin, and assayed aliquots of the medium at various intervals for lysosomal enzyme activity [115]. We find that there is a dramatic increase, after a lag period of 5 to 6 h, in the secretion of proteolytic enzymes into the medium of the tunicamycin-treated cells relative to untreated culture as shown in Figure 6. The proteolytic activity is tentatively identified as cathepsin B based on 1) substrate specificity (benzoyl-prolyl-L-phenylalanyl-L-arginyl-^{14}C-analine is rapidly hydrolyzed) [113], 2) the pH optimum for the reaction of 5.5 [113], and 3) the inhibition of the reaction by leupeptin [114] as shown in Figure 7. We have preliminary evidence that other lysosomal enzyme activities (β-hexosaminidase and β-glucuronidase) are also secreted into the medium of tunicamycin-treated cells.

The secretion of lysosomal enzymes by tunicamycin-treated cells is consistent with the findings of Sly and co-workers [111,112] that the carbohydrate moiety of these glycoproteins is required for their intracellular retention. The release of such large quantities of proteolytic enzymes may partially be responsible for the decreased amounts of membrane or secretory protein in tunicamycin-treated cultures. Since nonglycosylated proteins are more sensitive to proteases [35,40,59,61,68,69,72,80,83,], they may be significantly degraded at pH 7.4 (the physiological pH of culture media) even though the radiolabeled substrate is not hydrolyzed very rapidly at this pH (see Fig. 7). It is also possible that the secreted lysosomal proteases are

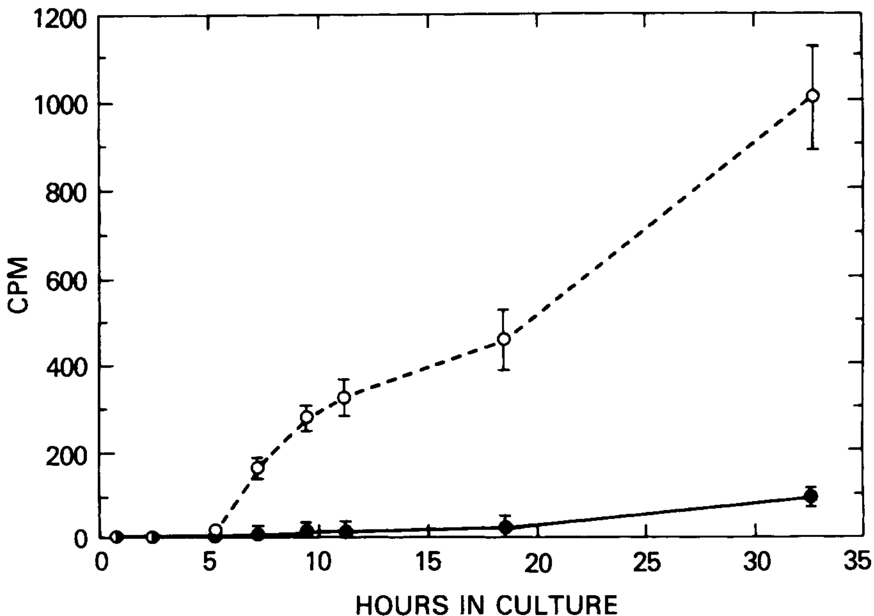

Fig. 6. Effect of tunicamycin on the secretion of proteolytic enzymes into the medium. Chick embryo fibroblasts were incubated in serum-free medium containing polyvinylpyrrolidone-40, with or without tunicamycin (0.05 μg/ml). Aliquots of the medium were assayed at various intervals for lysosomal enzyme activity as described in Methods. Medium of control cells (closed circles), medium of tunicamycin-treated cells (open circles).

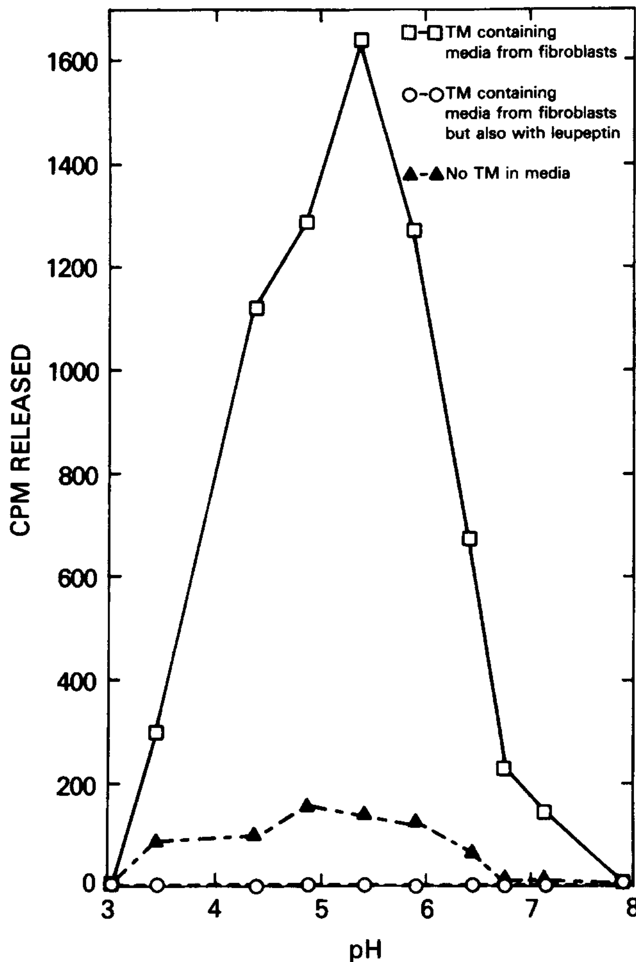

Fig. 7. Effect of pH and leupeptin on the proteolytic activity released in the medium after tunicamycin treatment. Medium of control or tunicamycin (0.05 μg/ml)-treated cells was prepared as in legend of Figure 7. The proteolytic activity was assayed as described in Methods, in presence or absence of leupeptin (50 uM), the pH ranging from 3 to 8.

cotransported to the surface with membrane or secretory proteins; hence, degradation might be initiated during intracellular processing.

CONCLUSIONS

The carbohydrate moieties of glycoproteins may serve as signals in the fusion and trafficking of vesicles in export of cellular products or in the extensive recycling of membranes. Such a signaling mechanism would allow for the efficient transport of glycoproteins from one organelle to another. The potential number of "code words" specified by the carbohydrate constituents of glycoproteins are numerous, taking into account the number of possible structural rearrangements within a single

carbohydrate chain. We envision that secretory and lysosomal proteins are released in the cisternae of the endoplasmic reticulum and are subsequently cotransported to the Golgi. However, once in the Golgi, the lysosomal hydrolases are selectively removed by binding to monovalent carbohydrate-binding proteins in differentiated regions of the Golgi (GERL). The differentiated regions give rise to transport vesicles, containing exclusively lysosomal enzymes that ultimately fuse with preexisting lysosomes or differentiate into lysosomes. The secretory products become enclosed in membrane vesicles destined to become plasma membrane. The soluble content of these vesicles is released outside when the transport vesicles fuse with the plasma membrane by exocytosis.

In several respects, tunicamycin treatment of fibroblasts appear to mimic the secretory characteristics of fibroblasts from patients with I-cell disease in that both secrete lysosomal enzymes. I-cell fibroblasts do not synthesize the mannosyl-PO_4 "sorting signal" because of a genetic defect [110] and tunicamyicin-treated fibroblasts do not synthesize N-glycosidically linked oligosaccharides [36–39]. Treatment of fibroblasts with the lysosomotrophic amine chloroquine also greatly enhances the secretion of newly synthesized acid hydrolases [112]. The mechanism is different in this case since "high uptake" forms of the enzymes are released which contain the "sorting signal." There is evidence that chloroquine treatment prevents the release of bound enzyme from the lectin receptor and thus depletes the cell of free lectin for additional binding [112]. Apparently both inactivation of the lectin for Man-6-P and loss of the Man-6-P "sorting signal" produces a similar result, secretion of lysosomal acid hydrolases into the medium.

The findings that carbohydrate is not required for secretion per se suggest that secretory proteins can be exported by nonspecific vesicles. Although in most of these studies, some impairment in intracellular processing was observed. The carbohydrate component of secretory glycoproteins may serve to restrict their transport to one population of vesicles, providing an efficient vehicle of export. This may be important because many vesicle populations shuttle to and from the plasma membrane. In addition, some secretory glycoproteins function in specific extracellular spaces relative to the cell. In such cases it might be advantageous for the cell to package and ship the glycoprotein to a specific region of the plasma membrane for release. An example of such a protein is the acetylcholine esterase, a secretory glycoprotein that functions in the extracellular junctions between neurons and muscles. In fact, we have shown that nonglycosylated fibronectin appears to have an intracellular distribution different from that of the glycosylated species [40].

Further investigations will be required to elucidate the role of carbohydrates in intracellular processing and secretion of glycoproteins. While carbohydrates may not be required for export per se, they may serve as "code words" responsible for the "routing" of glycoproteins in the intracellular membrane systems.

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