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Evidence for role of glycoprotein carbohydrates in membrane transport: Specific inhibition by tunicamycin

(glycosylation/glucose transport/amino-acid transport/plasma membrane/glucose-regulated proteins)

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ABSTRACT **Using tunicamycin, we have investigated the role of glycoproteins in membrane transport. Tunicamycin is a glucosamine-containing antibiotic that specifically inhibits dolichol pyrophosphate-mediated glycosylation of asparaginyl residues of glycoproteins. Inhibition of protein glycosylation in chick embryo fibroblasts by tunicamycin or other inhibitors of glycosylation resulted in defective transport of glucose, uridine, and amino acid analogs (α-aminoisobutyrate and cycloleucine). The defect in glucose transport is accompanied by decreased glucose metabolism, as determined by rates of CO_2 and lactate production. In contrast, tunicamycin treatment did not affect other membrane-associated processes, such as secretion of fibronectin and procollagen, uptake of glucose by passive diffusion, Na^+/K^+ ATPase and adenylate cyclase activities, or stimulation of adenylate cyclase by prostaglandin and cholera toxin. Two glucose/glycosylation-regulated membrane proteins with apparent subunit molecular weights of 95,000 and 75,000 were induced by tunicamycin treatment. Our results indicate that glycoprotein glycosylation is required for membrane transport.**

The oligosaccharides on glycoproteins appear to play important roles in protein secretion (1), specific recognition of serum glycoproteins (2), protection of glycoproteins against proteolytic degradation (3), the insertion or proper orientation of glycoproteins in the plasma membrane (4, 5), and cell adhesion and morphology (4–6). Because the transport of metabolites across cell membranes is thought to be mediated via glycoprotein carriers (7), we examined whether inhibiting protein glycosylation would alter a number of plasma membrane enzymatic and nutrient transport processes.

For these studies we have used tunicamycin, an inhibitor of the synthesis of *N*-acetylglucosaminyl pyrophosphoryl polyisoprenol (8–10). Because this reaction is required for the synthesis of the core sequence of *N*-glycosidically linked oligosaccharides, tunicamycin treatment results in synthesis of glycoproteins deficient in asparagine-linked oligosaccharides (10).

We present evidence that inhibition of protein glycosylation results in defective membrane transport and glucose metabolism in chick embryo fibroblasts (CEF). We demonstrate that the carbohydrate moieties of glycoproteins are required for the normal transport of metabolites across the plasma membrane and their subsequent metabolism. This effect might occur directly on carrier function or indirectly by inhibiting insertion of transport molecules into the membrane or by increasing their degradation rate (3).

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MATERIALS AND METHODS

Cell Culture. Secondary or tertiary CEF were grown for 24 hr in the presence or absence of tunicamycin (0.05 μg/ml or 50 nM) in 35-mm plastic tissue culture dishes (Costar) as described (11).

Transport Assays. For uptake experiments, the conditioned medium was aspirated and fresh medium containing 0.05 μg of tunicamycin per ml and the radiolabeled transport substrate was added. After incubation at 23°C for the times indicated, the labeling medium was decanted and cells were rapidly washed three times with ice-cold Dulbecco's phosphate-buffered saline containing calcium and magnesium.

Free intracellular radiolabeled substrate was extracted with ice-cold 10% trichloroacetic acid. The radioactivity in this acid-soluble fraction was determined by liquid scintillation spectrometry. Alternatively, cultures were homogenized in 2% sodium dodecyl sulfate ($NaDodSO_4$)/10 mM sodium phosphate, pH 7.0, and heated for 2 min at 100°C; then total radioactivity was determined. The results of these two methods were identical.

For efflux experiments, the cells were preloaded with substrate by incubation with 2 μCi (74 MBq) of α-amino[^{14}C]isobutyrate per ml in regular medium with 0.05 μg of tunicamycin per ml for 2 hr at 37°C. The labeling medium was poured off, cells were washed rapidly three times with phosphate-buffered saline equilibrated to 37°C, and fresh medium containing 0.05 μg of tunicamycin per ml was added. After incubation at 23°C for 10 min, the culture medium was assayed for radioactivity. At time zero, the control and tunicamycin-pretreated cultures contained approximately equivalent amounts of trichloroacetic acid-soluble radioactivity per μg of cell protein.

Na^+/K^+ ATPase and Adenylate Cyclase Assays. Membranes for Na^+/K^+ Mg^{2+} ATPase assays were prepared as described (12) from cells grown for 24 hr in medium with or without 0.2 μg of tunicamycin per ml. Membranes for adenylate cyclase assays were isolated as described (13). To investigate cholera toxin stimulation, we further preincubated cells for 4 hr with or without tunicamycin in serum-free medium to which 10 ng of cholera toxin per ml was added 3 hr before isolation of the membranes.

Na^+/K^+ Mg^{2+} ATPase activity was assayed by determining the rate of phosphate liberation from ATP; Mg^{2+} ATPase activity was determined by substituting 1 mM ouabain for the 3.3 mM KCl; and Na^+/K^+ ATPase activity was calculated as the difference between total ATPase and Mg^{2+} ATPase activities

Abbreviations: CEF, chick embryo fibroblasts; $NaDodSO_4$, sodium dodecyl sulfate; AIB, α-aminoisobutyric acid; GRP, glucose/glycosylation regulated protein.

(12). Adenylate cyclase activity was assayed by determining the rate of formation of cyclic [^{32}P]AMP from [α-^{32}P]ATP as described (14). Inorganic phosphate was determined by the procedure of Ames *et al.* (15).

Glucose Utilization. The rate of glucose metabolism was measured by a modification of a previous procedure (16) by determining the amount of $^{14}CO_2$ released in 30-min assays with D-[*U*-^{14}C]glucose as substrate. Cultures in medium containing 0.05 μg of tunicamycin per ml were placed in an airtight chamber. The reaction was initiated by adding radiolabeled glucose with a 25-gauge hypodermic needle through a resealable port. The reaction was terminated by adding concentrated sulfuric acid. The respiration chamber was pressurized to force the liberated CO_2 out of the chamber into a Hyamine hydroxide trap. In control experiments to determine the efficiency of CO_2 recovery, we were able to capture more than 97% of the CO_2 added as sodium [^{14}C]bicarbonate (Amersham).

Other Procedures. Cells were homogenized and electrophoresed in $NaDodSO_4$/polyacrylamide slab gels (3, 11). Protein was determined by the Lowry procedure (17), with bovine serum albumin standards.

Chemicals. Tunicamycin was a gift from Gakuza Tamura via the Drug Evaluation Branch of the National Cancer Institute (lot no. 177382). L-[*U*-^{14}C]Leucine (specific activity 325 Ci/mol), D-[*U*-^{14}C]glucosamine (238 Ci/mol), [*U*-^{14}C]uridine (462 Ci/mol), 2-deoxy-D-[1-^{14}C]glucose (54 Ci/mol), D-[*U*-^{14}C]glucose (213 Ci/mol), L-[1-^{3}H(N)]glucose (12 Ci/mmol), D-[2-^{3}H(N)]mannose (18 Ci/mmol), α-amino[1-^{14}C]isobutyric acid (52 Ci/mol), and [1-*carboxyl*-^{14}C]aminocyclopentane-1-carboxylic acid (cycloleucine, 30 Ci/mol) were obtained from New England Nuclear.

RESULTS

We first examined the effects of tunicamycin treatment on the rates of carrier-mediated uptake of radioactive 2-deoxy-D-glucose, uridine, cycloleucine, 3-*O*-methyl-D-glucose, and α-aminoisobutyric acid (AIB) at 23°C for up to 15 min. The initial rates of transport of 2-deoxy-D-[^{14}C]glucose, [^{14}C]uridine, and [^{14}C]AIB were decreased to 25–50% of the control value after 24 hr of treatment with tunicamycin (Fig. 1). Similar results were obtained with [^{14}C]cycloleucine or 3-*O*-methyl-D-[^{14}C]glucose as transport substrate (results not shown). Differences of 4-fold (629 compared to 2397 cpm/μg per 10 min) were also found in the rates of efflux of AIB between untreated control and tunicamycin-treated cultures. In contrast, the rate of L-[^{3}H]glucose uptake by passive diffusion was not affected by tunicamycin pretreatment (results not shown).

In these experiments, the effects of tunicamycin on glycosylation were monitored by D-[2-^{3}H]mannose and D-[^{14}C]-glucosamine incorporation into total trichloroacetic acid-precipitable material for 18 hr; protein synthesis was measured by [^{14}C]leucine incorporation. Mannose incorporation was decreased by 95% in the tunicamycin-treated cultures, glucosamine by 69%, and leucine by only 20%. Cell viability was unaffected, as determined by trypan blue exclusion (97% for control and tunicamycin-treated cells). We emphasize that all incorporation and uptake assays were performed in complete culture medium in an attempt to measure only physiologically significant alterations.

We examined the decrease in glucose uptake in more detail. The inhibition required pretreatment of cells. When CEF were pretreated with tunicamycin for various intervals, the uptake rates of 2-deoxy-D-glucose declined roughly linearly over 24 hr (Fig. 2). Addition of tunicamycin directly to control cells without pretreatment did not alter rates of 2-deoxy-D-glucose uptake (Fig. 2). Therefore, tunicamycin does not appear to be a direct inhibitor of glucose transport, in contrast to phlorizin or cytochalasin B (18). This slow decline suggests that tunicamycin might act via a cumulative, structural mechanism, such as decreased numbers of effective glucose carriers. This suggestion is supported by the finding that tunicamycin treatment decreases the V_{max} for 2-deoxy-D-glucose uptake by 50–75% without altering the K_m (Fig. 3).

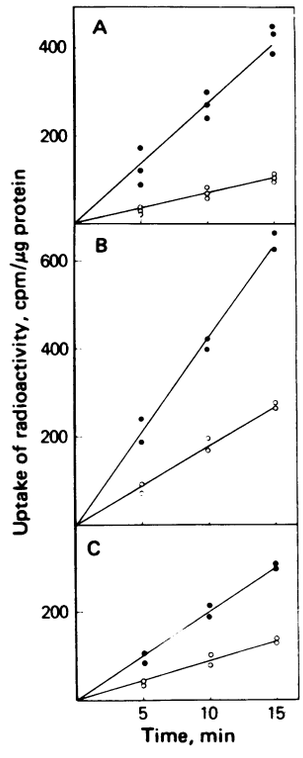

FIG. 1. Uptake of 2-deoxy-D-glucose (*A*), uridine (*B*), and AIB (*C*). All points in this and subsequent figures are the average of triplicate determinations. Similar experiments to determine rates of rubidium ($^{86}Rb^{+}$) uptake yielded variable results, with inhibitions of uptake by tunicamycin pretreatment ranging from 0 to 50%. ●, Without tunicamycin; ○, with tunicamycin.

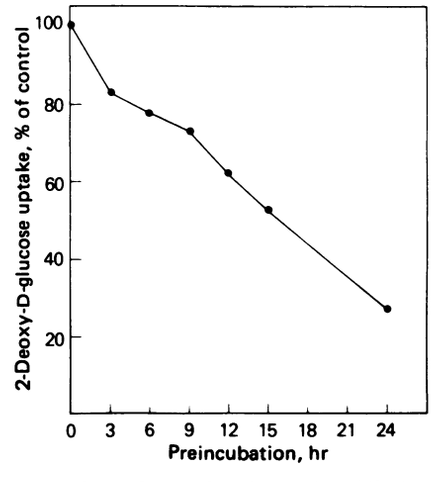

FIG. 2. Time dependence of tunicamycin inhibition of 2-deoxy-D-glucose uptake. Cells were grown in the presence or absence of 0.05 μg of tunicamycin per ml for the indicated times and uptake of 2-deoxy-D-glucose was assayed. In this experiment there was no inhibition of [^{14}C]leucine incorporation into total protein at 3 and 6 hr.

Table 1. Effect of tunicamycin pretreatment on the rate of lactate and CO_2 production

Conditions	nmol released/30 min per mg protein	
	$^{14}CO_2$	Lactate
Control	41	327
Tunicamycin-treated	10	71

Glucose metabolism was measured as described in *Materials and Methods* and also determined by measuring the rate of lactate production (19).

The inhibitory effects of 24-hr treatment with tunicamycin on 2-deoxy-D-glucose uptake were completely reversed after 24 hr of culturing cells in the absence of tunicamycin.

Tunicamycin does not appear to act by inhibiting hexokinase and hexose phosphorylation since the uptake of 3-*O*-methyl-D-[^{14}C]glucose (a glucose analogue that is not phosphorylated) was also inhibited 50–75% in 0.5 to 2-min uptake assays. However, tunicamycin pretreatment does decrease the rate of glucose utilization (Table 1). The rate of release of $^{14}CO_2$ from D-[*U*-^{14}C]glucose (specific activity 213 Ci/mol; 0.5 μCi/ml) was decreased to 25–50% of its original value in 30-min assays at 23°C after 24 hr of treatment with 0.05 μg of tunicamycin per ml. A second parameter of glucose utilization is cellular lactate production. The rate of lactate production, measured as described (19), was also substantially decreased after tunicamycin treatment (Table 1).

We next examined the specificity of tunicamycin's effects on other plasma membrane processes (Table 2). After 24 hr of treatment with a dose as high as 0.25 μg of tunicamycin per ml (0.25 μM), the enzymatic activities of total ATPase and of ouabain-sensitive Na^+/K^+ ATPase in crude membranes were not significantly altered (Table 2). Basal and fluoride-stimulated adenylate cyclase activities were also unaffected by tunicamycin pretreatment. Although adenylate cyclase activation by GTP alone may have been decreased, the activation of adenylate cyclase by GTP plus prostaglandin E_1 was similar.

Tunicamycin treatment for 24 hr also did not affect the response of CEF to cholera toxin (Table 2). The binding of cholera toxin to cells is apparently dependent on the specific oligosaccharide unit found on the ganglioside GM_1, which is not related to the tunicamycin-sensitive asparagine-linked oligosaccharide (20). The subsequent activation of adenylate cyclase by cholera toxin involves the transfer of a protein subunit across the plasma membrane (21).

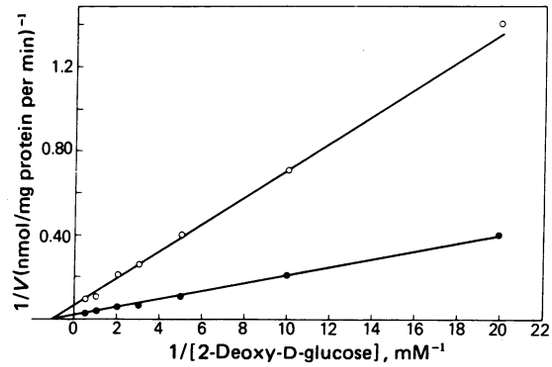

FIG. 3. Effect of tunicamycin on the transport kinetics of 2-deoxy-D-glucose. Cells were grown in the presence or absence of 0.05 μg of tunicamycin per ml for 24 hr. After cells were washed with phosphate-buffered saline, fresh glucose-free medium was added containing various concentrations of deoxy-D-[^{14}C]glucose and unlabeled 2-deoxy-D-glucose (0.01–5 mM). The ratio of labeled to unlabeled 2-deoxy-D-glucose was kept constant for each concentration (0.11 Ci/mol). K_m = 1 mM. ●, Control, V_{max} = 50; ○, with tunicamycin, V_{max} = 14.

We also examined whether other inhibitors of glycosylation besides tunicamycin would inhibit transport. Glucosamine and deoxyglucose would have competed with glucose in uptake assays; we therefore examined their effects on the uptake of AIB. D-Glucosamine (20 mM) and 2-deoxy-D-glucose (10 mM) inhibited the uptake of AIB into CEF. After 24 hr of treatment, both compounds inhibited AIB uptake by 50% in 10-min uptake assays. This finding provides further support for the suggestion that membrane transport requires glycosylated proteins.

Table 2. Adenylate cyclase and Na^+/K^+ ATPase activities in control and tunicamycin-treated cells

A. Membrane ATPase activity (μmol P_i/60 min per mg of membrane protein)

Conditions	Total (Na^+/K^+ Mg^{2+} ATPase)	Na^+/K^+ ATPase (ouabain-inhibited)
Control	8.14	1.75
Tunicamycin	8.28	1.78
Boiled membranes	0.02	0.00

B. Adenylate cyclase activity (pmol/10 min per mg of membrane protein)

Conditions	Control	Relative stimulation over basal	Tunicamycin	Relative stimulation over basal
No addition (basal)	39	—	41	—
GTP	128	3.3	95	2.3
GTP + prostaglandin E_1	196	5.0	157	3.8
Fluoride	573	14.7	617	15.1

C. Cholera toxin (CT) stimulation of adenylate cyclase (pmol/10 min per mg of membrane protein)

Conditions	Control		Relative stimulation over basal	Tunicamycin		Relative stimulation over basal
	−CT	+CT		−CT	+CT	
No addition (basal)	25	125	5.1	19	91	4.7
GTP	106	337	13.5	42	242	12.5

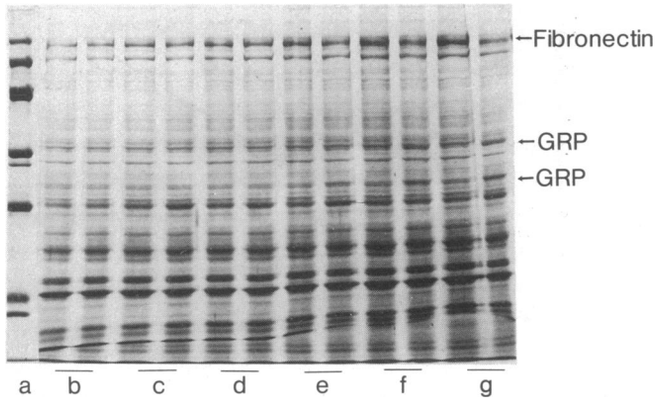

FIG. 4. Time dependence of effects of tunicamycin on protein-staining patterns of whole-cell homogenates subjected to polyacrylamide gel (7.5%) electrophoresis. Shown are Coomassie blue-stained gels of (a) standard proteins (chicken gizzard filamin, 240,000 daltons; rabbit skeletal muscle myosin, 200,000 daltons; RNA polymerase β and β' subunits, 150,000 and 160,000 daltons, respectively; phosphorylase a, 94,000 daltons; RNA polymerase α subunit, 90,000 daltons; bovine serum albumin, 68,000 daltons; ovalbumin, 43,000 daltons; and RNA polymerase σ subunit, 39,000 daltons) and homogenates of cells treated with tunicamycin for (b) 0 hr, (c) 3 hr, (d) 6 hr, (e) 9 hr, (f) 12 hr, and (g) 24 hr. Cells were homogenized in equal aliquots (250 μl) of 2% $NaDodSO_4$, containing 2 mM phenylmethylsulfonyl fluoride to inhibit proteases, and 0.1 M dithiothreitol. Equal aliquots (50 μl) of each homogenate were applied to the gel. GRP, glucose/glycosylation regulated protein.

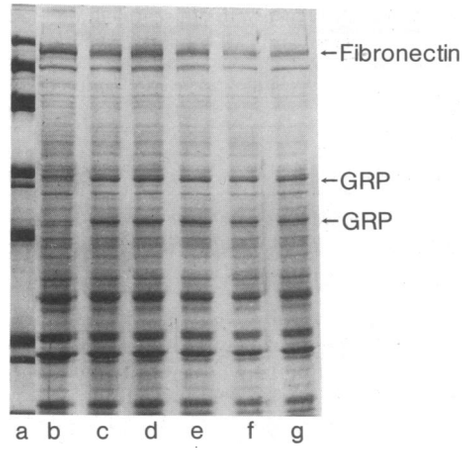

FIG. 5. Effect of tunicamycin treatment on protein-staining patterns of cells grown in medium containing various concentrations of glucose. Cells were homogenized and electrophoresed as described in the legend to Fig. 4. Shown are Coomassie blue-stained gels of (a) standard proteins, (b) control homogenate, and homogenates of tunicamycin-treated cells grown in medium containing (c) 5 mM, (d) 10 mM, (e) 20 mM, (f) 50 mM, and (g) 100 mM glucose.

However, studies with these two compounds must be interpreted with extreme caution because they cause marked inhibition of protein and nucleic acid synthesis (greater than 50%) at the concentrations required to inhibit glycosylation by 99% (22).

To determine the effects of tunicamycin on individual cell proteins, we treated cells with the drug for various times for up to 24 hr, homogenized them in 2% $NaDodSO_4$, and analyzed them by electrophoresis in polyacrylamide gels. The patterns of protein staining in gels of homogenates of tunicamycin-treated cultures were similar to those of untreated control cultures, with the exception of three bands (see Figs. 4 and 5).

After 24 hr of pretreatment with tunicamycin, there was a significant decrease in the fibronectin band, as we had reported previously (3), but the most apparent alterations were the marked increases of two major polypeptide bands of apparent molecular weights of 95,000 and 75,000 in homogenates of tunicamycin-treated cultures. The electrophoretic mobilities of these bands are identical to those of the "glucose/glycosylation regulated proteins" (GRP) induced in chick cells subjected to glucose starvation or inhibition of glycosylation (23, 24).

We investigated whether the induction of the GRP during tunicamycin treatment was a result of glucose deprivation caused by the 50–75% decreases in glucose uptake. Intracellular levels of glucose were raised by maintaining tunicamycin-treated cells in unusually high concentrations of glucose, which can enter cells by passive diffusion. The increases in intracellular glucose were confirmed by determining the amounts of tracer 3-*O*-methyl-D-[^{14}C]glucose (2 μCi/mmol of glucose) present intracellularly after 15 min of incubation in media containing normal or elevated concentrations of glucose. In the highest concentration of glucose (100 mM), the tunicamycin-treated cells contained over 10-fold more intracellular radioactivity than cells maintained in normal medium (5 mM glucose). Nevertheless, the 95,000- and 75,000-dalton GRPs were induced regardless of the external concentration of glucose (Fig. 5), indicating that they are induced via mechanisms other than glucose deprivation.

DISCUSSION

The principal finding of this study on the requirement for glycoproteins in membrane transport is that inhibition of protein glycosylation in CEF by tunicamycin or other inhibitors of glycosylation results in defective transport of glucose, uridine, and two amino acid analogs (AIB and cycloleucine). In contrast, tunicamycin treatment does not affect other membrane-associated processes, such as the secretion of fibronectin and procollagen (3, 25) and the enzymatic activities of Na^+/K^+ ATPase and adenylate cyclase or the stimulation of adenylate cyclase by prostaglandin and cholera toxin.

The simplest explanation for the effects of tunicamycin on nutrient transport is that insufficient glycosylation results in fewer active carrier molecules. The progressive loss of total transport activity by cells would be the result of the turnover of previously synthesized carriers and dilution due to cell growth. However, a better knowledge of the functional activity of fibroblast membrane transport proteins isolated from control and tunicamycin-treated cells will be necessary to establish how tunicamycin acts. For example, inhibition of glycosylation could directly interfere with the carrier function of transport molecules or it might result in their faulty insertion into the membrane or in increased rates of destruction due to increased proteolytic susceptibility (3–5, 26).

It is conceivable that the effects of tunicamycin on transport processes are a secondary consequence of growth inhibition. However, this possibility seems unlikely since (*i*) the inhibition is not marked over the 24-hr test period (15–20% inhibition of total protein synthesis); (*ii*) protein synthesis was not affected at all before 6 hr (3), whereas effects on transport were already apparent by 3 hr (Fig. 2); (*iii*) 5-fold higher concentrations of tunicamycin, which inhibited protein synthesis to a greater extent (25–30%), did not result in a further decrease in uptake at 24 hr; (*iv*) equivalent inhibition of protein synthesis with low concentrations of cycloheximide (0.005 μg/ml) did not decrease the rates of 2-deoxy-D-glucose uptake per μg of cell protein; and (*v*) the effects on transport processes were not accompanied by effects on other membrane functions that can change with growth state (Table 2).

In addition, it seems unlikely that the observed decrease in transport is due to selective inhibition of protein synthesis of glycoproteins involved in transport since tunicamycin does not prevent synthesis of other glycoproteins. For example, earlier studies with fibronectin, a well-characterized membrane glycoprotein, showed that tunicamycin treatment completely blocked its glycosylation but did not inhibit its synthesis and secretion (3). The decrease in cell-associated fibronectin was due to enhanced degradation of the nonglycosylated protein (3). Similarly, tunicamycin did not inhibit procollagen synthesis (3, 25), and inhibition of glycosylation of the immunoglobulin light chain in myeloma cells also did not inhibit the synthesis of the protein moiety of the glycoprotein (27).

The simultaneous decrease in both transport and metabolism of glucose suggests that transport may be rate limiting at low concentrations (5 mM or less), as has been suggested (28), although we cannot completely exclude a possible direct effect of tunicamycin on cellular glucose metabolism.

The 95,000- and 75,000-dalton GRPs induced by tunicamycin treatment may be involved in glucose transport (23) since a recent report claims that proteins of similar apparent molecular weights may mediate glucose transport in fat cells (29). Yet we find that, even though these proteins are induced by tunicamycin, glucose transport is decreased. This finding suggests either that these GRPs are not involved in glucose transport or that, if they are involved, they must be glycosylated in order to function. The 95,000-dalton GRP is the nonglycosylated precursor of a 97,000-dalton protein normally present in the plasma membrane (23, 30).

Because of its effects on nutrient transport and glucose utilization, tunicamycin's effects on cell behavior should be interpreted with caution. However, pools of some transported metabolites are not altered; for example, total soluble pools of radioactive [^{14}C]glucosamine and $^{35}SO_4^{2-}$ are identical in control and tunicamycin-treated cells after 24 hr of labeling (3, 31).

Are all of tunicamycin's effects a direct result of inhibiting glycosylation? The effects of treatment of CEF by this antibiotic on quantities and turnover rates of the major cell-surface protein fibronectin (3), on the synthesis of fibroblastic sulfated proteoglycans (31), and on membrane transport processes (this paper) all correlate with the expected presence of a carbohydrate moiety on proteins. In addition, other inhibitors of glycosylation also mimic tunicamycin's effects on membrane transport.

A recent report (32) on the inhibitory effects of wheat germ agglutinin on transport in several cell lines may be relevant to the present study since this lectin binds to *N*-acetylglucosamine, which is present in the same asparagine-linked class of oligosaccharide that is affected by tunicamycin.

In conclusion, the transport of three major classes of nutrient is inhibited by inhibition of protein glycosylation. This finding suggests that glycosylation of membrane carrier glycoproteins is necessary for several membrane transport processes, perhaps to permit their correct insertion or maintenance in the plasma membrane or to protect against abnormally rapid proteolytic degradation (3, 26).

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