The relationship between glycosylation and glycoprotein metabolism of mouse neuroblastoma N18 cells

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Two inhibitors of glycosylation, glucosamine and tunicamycin, were utilized to examine the effect of glycosylation inhibition in mouse neuroblastoma N18 cells on the degradation of membrane glycoproteins synthesized before addition of the inhibitor. Treatment with 10 mM-glucosamine resulted in inhibition of glycosylation after 2 h, as measured by [^3H]fucose incorporation into acid-insoluble macromolecules, and in a decreased rate of glycoprotein degradation. However, these results were difficult to interpret since glucosamine also significantly inhibited protein synthesis, which in itself could cause the alteration in glycoprotein degradation [Hudson & Johnson (1977) *Biochim. Biophys. Acta* **497**, 567–577]. N18 cells treated with 5 μg of tunicamycin/ml, a more specific inhibitor of glycosylation, showed a small decrease in protein synthesis relative to its effect on glycosylation, which was inhibited by 85%. Tunicamycin-treated cells also showed a marked decrease in glycoprotein degradation in experiments with intact cells. The inhibition of glycoprotein degradation by tunicamycin was shown to be independent of alterations in cyclic AMP concentration. Polyacrylamide-gel electrophoresis of isolated membranes from N18 cells, double-labelled with [^{14}C]fucose and [^3H]fucose, revealed heterogeneous turnover rates for specific plasma-membrane glycoproteins. Comparisons of polyacrylamide gels of isolated plasma membranes from [^3H]fucose-labelled control cells and [^{14}C]fucose-labelled tunicamycin-treated cells revealed that both rapidly and slowly metabolized, although not all, membrane glycoproteins became resistant to degradation after glycosylation inhibition.

One of the interesting problems of cell biology is the nature of events involved in the metabolic maintenance of plasma-membrane components, including membrane glycoproteins. Work has shown that these macromolecules undergo active metabolism that includes heterogeneous rates of synthesis and degradation (Kaplan & Moskowitz, 1975; Landry & Marceau, 1975; Tweto & Doyle, 1975; Mathews *et al.*, 1976; Hudson & Johnson, 1977*b*; Baumann & Doyle, 1978; Milenkovic *et al.*, 1978; Kaplan *et al.*, 1979). Current experiments on the synthesis of immunoglobulins and viral membrane glycoproteins support a model for glycoprotein synthesis in which nascent polypeptide chains on membrane-bound ribosomes are inserted into the endoplasmic reticulum and migrate to the plasma membrane through the system of intracellular membranes, undergoing glycosylation en route (Blobel & Doberstein, 1975; Katz *et al.*, 1977; Lingappa *et al.*, 1978, 1979; Parry, 1978; Plough *et al.*, 1979). Much less is known about the

mode of degradation and turnover of membrane glycoproteins once they are inserted in the cell surface. There is some evidence that membrane components undergo endocytosis and subsequent degradation in lysosomes (Ascoli & Puett, 1978; Das & Fox, 1978; Parry, 1978). Alternatively, turnover of membrane glycoproteins may be mediated by proteinases and glycosidases, which have been shown to exist at the cell surface, as well as shedding of cell-surface components into the surrounding medium (Patt & Grimes, 1974; Parry, 1978).

There has been increasing evidence of late that the oligosaccharide portion of glycoproteins plays an important role in the postsynthetic processing of certain membrane glycoproteins. Studies with glycosylation-inhibited mutants of cultured cell lines or glycosylation inhibitors, such as glucosamine, 2-deoxyglucose or tunicamycin, have revealed that glycoproteins lacking oligosaccharide may be altered in their insertion into plasma membranes, their

conformation within the plasma membrane, or their rates of degradation (Eagon & Heath, 1977; Leavitt *et al.*, 1977; Pouyssegur & Pastan, 1977; Morrison *et al.*, 1978; Olden *et al.*, 1978, 1979; Pouyssegur & Yamada, 1978; Gibson *et al.*, 1979). For example, inhibiting glycosylation of either viral glycoproteins or immunoglobulins allows biosynthesis to proceed normally, but interferes with the final insertion of the incomplete glycoprotein into plasma membranes or release at the cell surface (Eagon & Heath, 1977; Hickman *et al.*, 1977; Leavitt *et al.*, 1977; Morrison *et al.*, 1978; Gibson *et al.*, 1979). In mouse 3T3 cells, an inhibition of glycosylation of plasma-membrane glycoproteins, including the glucose-regulated proteins, results in an altered conformation of these glycoproteins in plasma membranes, as measured by altered susceptibility to external iodination by lactoperoxidase (Pouyssegur & Pastan, 1977; Pouyssegur & Yamada, 1978). The large-external-transformation-sensitive (LETS) glycoprotein is synthesized in the presence of tunicamycin and is available on the cell surface of chick embryo fibroblasts, but the non-glycosylated LETS glycoprotein appears to be more sensitive to degradation, both *in vivo* and *in vitro* (Olden *et al.*, 1978, 1979).

We have previously found that inhibition of cell growth or protein synthesis can also alter the metabolism of cell-surface glycoproteins. The addition of dibutyryl cyclic AMP (N^6O^2' -dibutyryl adenosine 3':5'-cyclic monophosphate) and theophylline to neuroblastoma cell cultures inhibited both cell growth and the degradation of cell-surface glycoproteins (Milenkovic *et al.*, 1978). Inhibition of translational events, mediated by puromycin or cycloheximide, has also been shown to inhibit the metabolic turnover of plasma-membrane glycoproteins (Hudson & Johnson, 1977b). The present study was carried out to investigate the possible relationship between the synthesis of the oligosaccharide moiety of cell glycoproteins and the degradation or turnover of glycoproteins previously inserted in the plasma membrane.

Materials and methods

Materials

Radioactive fucose was purchased from New England Nuclear (Boston, MA, U.S.A.) and radioactive leucine from Amersham (Arlington Heights, IL, U.S.A.). Cell culture components were from Grand Island Biological Company (Grand Island, NY, U.S.A.). Non-radioactive fucose and glucosamine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); tunicamycin was kindly given by Dr. G. Tamura (Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku, Tokyo, Japan).

Neuroblastoma cell growth

The N18 line of mouse neuroblastoma cells was obtained from Dr. B. Spooner (Division of Biology, Kansas State University) and maintained by the method of Hughes *et al.* (1979). Monolayer cultures were grown for at least 36 h after last transfer to allow recovery from trypsin treatment before addition of radioactive precursors or glycosylation inhibitors.

Incubation with glycosylation inhibitors and radioactive precursors

Glucosamine-mediated glycosylation inhibition was produced by incubation of monolayer cultures with 1–10 mM-D-glucosamine for various periods of time. To inhibit glycosylation via tunicamycin, N18 monolayer cultures were scraped with a 'rubber policeman', suspended in complete medium containing 5 μ g of tunicamycin/ml, and incubated at 37°C for 5 h. Untreated or glycosylation-inhibited cells were labelled with [3 H]fucose, [14 C]fucose or [3 H]leucine in complete medium plus or minus glycosylation inhibitors. The [3 H]fucose (sp. radioactivity 12.1 Ci/mmol) was diluted with unlabelled fucose and used at 1–5 μ Ci/ml (2.6–2.9 nmol/ml); [14 C]fucose (sp. radioactivity 53.2 Ci/mmol) and [3 H]leucine (sp. radioactivity 70 Ci/mmol) were used undiluted at 1 μ Ci/ml (18.8 nmol/ml) and 4 μ Ci/ml (57 pmol/ml) respectively. [3 H]Fucose was supplemented with 2.5 μ M-fucose to avoid the possibility that fucose was rate-limiting to cell metabolism (Hudson & Johnson, 1977a,b).

Fucose and leucine incorporation into whole cells

Neuroblastoma cells, grown in monolayer cultures or as suspensions in complete medium, were incubated with [3 H]fucose or [3 H]leucine for various time periods and assayed for the incorporation of the radioactive precursor into cellular macromolecules by trichloroacetic acid precipitation as previously described (Milenkovic *et al.*, 1978). Leucine incorporation for duplicate samples of N18 cells labelled for 20, 40 or 60 min was determined. Since leucine incorporation was linear with respect to time during this period, rates of protein synthesis could be determined by linear regression analysis of the data. Amounts of protein were determined by the method of Lowry *et al.* (1951).

Cyclic AMP determination

N18 cells (5×10^6) in complete medium or incubated in complete medium plus 5 μ g of tunicamycin/ml for 5 h were washed twice with 5 ml of saline (0.9% NaCl) and suspended in 1 ml of 10% trichloroacetic acid in 50 mM-sodium acetate buffer. After centrifugation at 500g for 5 min, the supernatant was extracted three times with 5 vol. of water-saturated diethyl ether at 25°C. The aqueous

phase was then assayed for cyclic AMP by the method of Harper & Brooker (1975).

Plasma-membrane isolation and gel electrophoresis

Plasma membranes were isolated by a modification of the Zn^{2+} method of Warren *et al.* (1966) as described by Milenkovic *et al.* (1978). Plasma-membrane glycoproteins were separated on the sodium dodecyl sulphate/polyacrylamide-gel system of Laemmli (1970). Radioactively labelled gels were frozen at $-80^{\circ}C$ and sliced into 1.0-mm fractions with a Bio-Rad gel divider (model 190). 3H and ^{14}C radioactivity in each slice was determined as previously described (Milenkovic *et al.*, 1978).

Results

To examine the nature of the relationship between synthesis and degradation of membrane glycoproteins, a method was sought that would primarily inhibit the glycosylation of cell glycoproteins. High concentrations of glucosamine, in the range 1–10 mM, have been commonly used for this purpose and shown to inhibit glycosylation events in cultured cells (Kaluza, 1975; Knowles & Person, 1976; Pouyssegur & Yamada, 1978). Concentrations of glucosamine up to 2 mM appeared to have little effect on glycosylation, whereas higher concentrations markedly decreased $[^3H]$ fucose incorporation. Since 10 mM-glucosamine exhibited the greatest inhibition of fucose incorporation after 4 h, further studies on glycosylation inhibition were conducted with this concentration. Since we were interested in the effect of glycosylation inhibition on the metabolic turnover of membrane glycoproteins, the effect of 10 mM-glucosamine on the degradation of glycoproteins fucosylated before glucosamine treatment was determined. N18 cells in monolayer culture in complete medium were incubated to steady-state incorporation levels (16 h) with $[^3H]$ fucose, washed and re-incubated in complete medium containing 40 mM-fucose. The specific radioactivity of the remaining cellular glycoproteins was then measured after various time periods (Fig. 1). On addition of glucosamine, degradation proceeded normally for 4 h, after which the rate of degradation decreased compared with that of the controls (cultures not incubated with glucosamine). Fucose (40 mM) had no effect on glycoprotein synthesis (results not shown).

Since inhibition of protein synthesis in neuroblastoma cells has previously been shown to inhibit glycoprotein degradation (Hudson & Johnson, 1977a), it was therefore important to ascertain whether glucosamine preferentially inhibited glycosylation without affecting polypeptide synthesis. Protein synthesis appeared to be inhibited

Fig. 1. Degradation of fucosylated macromolecules in glucosamine-treated neuroblastoma cells

Neuroblastoma monolayer cultures were incubated with $1 \mu Ci$ of $[^3H]$ fucose/ml supplemented with $2.5 \mu M$ -fucose in complete medium for 16 h. The cells were washed twice with non-radioactive medium and incubated in complete medium containing 40 mM-fucose with or without 10 mM-glucosamine. At the times indicated after the removal of $[^3H]$ fucose, duplicate dishes were assayed for acid-insoluble specific radioactivity according to the procedure in the Materials and methods section. Degradation was expressed as the percentage of the specific radioactivity just before removal of $[^3H]$ fucose; ●, cells incubated with 40 mM-fucose; ○, cells incubated with 40 mM-fucose plus 10 mM-glucosamine. The vertical bars denote S.E.M. for four experiments each performed in duplicate.

approx. 60–70% during the 3–7 h period (Table 1), which was similar to the amount of glycosylation inhibition during that time period.

Tunicamycin, a specific inhibitor of asparagine-linked oligosaccharide synthesis, has been shown to have a minimal effect on protein synthesis (Duksin & Bornstein, 1977; Hickman *et al.*, 1977; Olden *et al.*, 1978). Concentrations of tunicamycin of up to $2 \mu g/ml$ inhibited glycosylation by less than 50%. N18 cells incubated in complete medium containing $5 \mu g$ of tunicamycin/ml for 5 h exhibited glycosylation inhibition of 85% but protein-synthesis inhibition of only 35% as measured by $[^3H]$ fucose incorporation and the rate of $[^3H]$ leucine incorporation (Table 2). Thus, tunicamycin preferentially inhibited glycosylation with a relatively slight effect on protein synthesis.

Table 1. *Protein synthesis in glucosamine-treated cells*

Cultures of N18 cells were grown in complete medium or incubated in complete medium containing 10mM-glucosamine for 3 or 7h. The cells were subsequently divided into 1 ml portions containing approx. 1×10^6 cells; 8 μ Ci of [3 H]leucine was added to each, and duplicate samples were incubated at 37°C for 20, 40 or 60 min. The cells were subsequently washed and assayed for leucine incorporation as described in the Materials and methods section. The rate of protein synthesis was determined by linear regression analysis. The values in parentheses are correlation coefficients, where ± 1 is a perfectly linear correlation.

Sample	Preincubation time (h)	Rate of [3 H]leucine incorporation (c.p.m./mg of protein per min)	Inhibition (%)
Control	None	208.6 (0.984)	0
Glucosamine (10mM)	3	85.6 (0.954)	59.0
Glucosamine (10mM)	7	57.1 (0.822)	72.6

Table 2. *Effect of tunicamycin on glycosylation and protein synthesis*

Cultures of N18 cells were incubated with the specified concentration of tunicamycin for 5h, after which the cells were divided into 1 ml portions containing approx. 1×10^6 cells. To assay glycosylation, 8 μ Ci of [3 H]fucose/ml was added to duplicate samples, the cells were incubated at 37°C for 2h, washed with saline and assayed for [3 H]fucose incorporation as described in the Materials and methods section. Values represent means for duplicate samples $\pm$ range. To assay protein synthesis, 8 μ Ci of [3 H]leucine/ml was added to duplicate samples, which were then incubated at 37°C for 20, 40 or 60 min, then washed with saline and assayed for leucine incorporation as described in the Materials and methods section. The rate of protein synthesis was determined by linear regression analysis. The values in parentheses are correlation coefficients, where ± 1 is a perfectly linear correlation. Abbreviation: N.D., not determined.

Concentration of tunicamycin (μ g/ml)	$10^{-3} \times$ [3 H]Fucose incorporation (c.p.m./mg of protein)	Inhibition of glycosylation (%)	Rate of [3 H]leucine incorporation (c.p.m./mg of protein per min)	Inhibition of protein synthesis (%)
None	1.735 ± 0.005		N.D.	
1	1.620 ± 0.030	6.90	N.D.	
None	0.804 ± 0.023		128.4 (0.961)	0
2	0.463 ± 0.070	42.4	N.D.	
5	0.120 ± 0.036	85.1	83.6 (0.958)	34.9

To establish a link between glycosylation and degradation of glycoproteins suggested by the effect of glucosamine, the effect of the presence of tunicamycin on glycoprotein degradation of N18 cells was examined. N18 cells were incubated with [3 H]fucose for 16h at which time 5 μ g of tunicamycin/ml was added to half the cell cultures. After 5h at 37°C, control and tunicamycin-treated cells were washed free of [3 H]fucose and incubated in complete medium containing 40mM unlabelled fucose as described in the Materials and methods section. After various time periods, the specific radioactivity of the fucosylated glycoproteins was determined. As was found with glucosamine, inhibition of glycosylation by tunicamycin significantly decreased the rate of glycoprotein degradation in comparison with the controls (Fig. 2).

Since cyclic AMP has been shown to inhibit glycoprotein degradation in neuroblastoma cells (Milenkovic *et al.*, 1978), cyclic AMP concen-

trations were assayed in N18 cells incubated for 5h in complete medium plus or minus 5 μ g of tunicamycin/ml, as described in the Materials and methods section. Tunicamycin-treated cells contained 1.14 μ g of cyclic AMP/mg of cell protein; control cells had 1.13 μ g of cyclic AMP/mg of protein. Thus, inhibition of glycoprotein degradation after tunicamycin treatment was not mediated by alterations in the intracellular concentrations of cyclic AMP.

Having demonstrated a link between glycosylation and glycoprotein degradation in experiments with intact cells, we were interested in what effect inhibition of glycosylation might have on specific plasma-membrane glycoproteins. Therefore, experiments were performed to compare the metabolic turnover of cell-surface glycoproteins in N18 cells where glycosylation continued with that of cell-surface glycoproteins in cells where glycosylation was inhibited by tunicamycin. The turnover of

Fig. 2. Degradation of fucosylated glycoproteins in tunicamycin-treated neuroblastoma cells

Neuroblastoma monolayer cultures were incubated in complete medium containing $1\mu\text{Ci}$ of $[^3\text{H}]\text{fucose}/\text{ml}$ supplemented with $2.5\mu\text{M}$ -fucose for 16h. The cells were scraped with a 'rubber policeman' and divided into two portions. Tunicamycin ($5\mu\text{g}/\text{ml}$) was added to one preparation, after which both sets of cells were incubated for 5h. The cells were then washed twice with non-radioactive medium and incubated in complete medium containing 40mM -fucose. Cells previously treated with tunicamycin were continually incubated with $5\mu\text{g}$ of tunicamycin/ml. At times indicated after the removal of $[^3\text{H}]\text{fucose}$, duplicate samples were assayed for acid-insoluble specific radioactivity as described in the Materials and methods section. Degradation was expressed as the percentage of the specific radioactivity just before removal of $[^3\text{H}]\text{fucose}$. ●, Cells incubated with 40mM -fucose; ○, tunicamycin-treated cells incubated with 40mM -fucose. The vertical bars denote S.E.M. for three experiments each performed in duplicate.

plasma-membrane glycoproteins in N18 cells undergoing normal glycosylation was assayed by the following double-label experiments.

The cells were labelled with $[^{14}\text{C}]\text{fucose}$ for 16h, washed, re-incubated without fucose for 6h to deplete intracellular pools of $[^{14}\text{C}]\text{fucose}$ and subsequently labelled with $[^3\text{H}]\text{fucose}$ for 3h. Plasma membranes were isolated and electrophoresed on sodium dodecyl sulphate/polyacrylamide gels as described in the Materials

Fig. 3. Turnover of fucosylated glycoproteins in neuroblastoma plasma membranes

Neuroblastoma cells were incubated for 24h in complete medium containing $1\mu\text{Ci}$ of $[^{14}\text{C}]\text{fucose}/\text{ml}$, washed twice with fresh medium, re-incubated for 6h in complete medium without fucose, and then incubated for 3h in complete medium containing $5\mu\text{Ci}$ of $[^3\text{H}]\text{fucose}/\text{ml}$ supplemented with $2.5\mu\text{M}$ -fucose. Plasma membranes were isolated and electrophoresed, and radioactivity was determined as described in the Materials and methods section. The broken lines represent tolerance limits of variability where $P = 0.05$.

and methods section (Fig. 3). Fig. 3 depicts the $^3\text{H}/^{14}\text{C}$ ratio for each portion of the gel, and the broken lines represent limits of variability (Milenkovic *et al.*, 1978). Glycoprotein degradation and re-synthesis would result in a loss of ^{14}C and an increase of ^3H , that is, a high $^3\text{H}/^{14}\text{C}$ ratio. Therefore, ratios above the limits of variability signify relative rapid turnover, and those below the limits signify relatively slow turnover. Fucosylated glycoproteins of apparent high and low molecular weights appear to undergo rapid turnover, whereas glycoproteins of apparent mol.wts. between 80000 and 100000 appear to turn over slowly. This is consistent with previous observations of glycoprotein turnover of another neuroblastoma cell line, N2a (Hudson & Johnson, 1977b; Milenkovic *et al.*, 1978).

Because the incorporation of $[^3\text{H}]\text{fucose}$ into glycoproteins during the second stage of incubation would be greatly decreased in tunicamycin-treated cells, the method used to measure turnover in Fig. 3 could not be used for glycosylation-inhibited cells. Instead, a comparison was made of degradation of specific cell-surface glycoproteins in the presence or absence of tunicamycin.

Monolayer cultures of N18 cells were incubated with $[^{14}\text{C}]\text{fucose}$ for 16h, after which $5\mu\text{g}$ of tunicamycin/ml was added and the cells were incubated for 5h. A control set of N18 cultures was incubated with $[^3\text{H}]\text{fucose}$ for 21h. A portion was removed from both the tunicamycin-treated and

Fig. 4. *Relative degradation of glycoproteins from plasma membranes of normal and glycosylation-inhibited cells*

Monolayer cultures of neuroblastoma cells were incubated in complete medium containing either $1 \mu\text{Ci}$ of $[^{14}\text{C}]\text{fucose/ml}$ or $5 \mu\text{Ci}$ of $[^3\text{H}]\text{fucose/ml}$ (supplemented with $2.5 \mu\text{M}$ -fucose) for 16 h. The cells were then scraped and $5 \mu\text{g}$ of tunicamycin/ml was added to the $[^{14}\text{C}]\text{fucose}$ -labelled cells. Both sets of cells were subsequently incubated for 5 h, after which a portion was removed from each preparation and counted for radioactivity, to determine the $^3\text{H}/^{14}\text{C}$ ratio before glycoprotein degradation. The cells were washed twice with non-radioactive medium and re-suspended in complete medium containing 40 mM-fucose (plus $5 \mu\text{g}$ of tunicamycin/ml for ^{14}C -labelled cells) for 4 h. The two sets of cells were combined and plasma membranes were isolated and electrophoresed as described in the Materials and methods section. The gel was sliced and the $^3\text{H}/^{14}\text{C}$ ratio was determined for each slice. The upper and lower pairs of broken lines represent tolerance limits of variability around the mean $^3\text{H}/^{14}\text{C}$ ratios before and after incubation with 40 mM-fucose respectively, where $P = 0.05$ (Mathews *et al.*, 1976). The molecular-weight scale was determined by electrophoresis of molecular-weight standards as described.

control preparations to determine the mean ratio of $^3\text{H}/^{14}\text{C}$ before glycoprotein degradation. Then both control cells and glycosylation-inhibited cells were washed and incubated in medium containing 40 mM-fucose for 4 h. The two sets of cells were combined, plasma membranes were isolated and the membranes were electrophoresed on sodium dodecyl sulphate/polyacrylamide gels as described in the Materials and methods section (Fig. 4).

In Fig. 4 the open circle indicates the mean $^3\text{H}/^{14}\text{C}$ ratio before re-incubation with 40 mM-fucose, that is, the expected $^3\text{H}/^{14}\text{C}$ ratio for any glycoprotein if its rate of degradation is the same in the presence or absence of tunicamycin. If tunicamycin inhibited the rate of degradation of a membrane glycoprotein, the $^3\text{H}/^{14}\text{C}$ ratio would be

below the tolerance limits of variability (upper pair of broken lines) around this expected mean (open circle). The small closed circles represent $^3\text{H}/^{14}\text{C}$ ratios for membrane-glycoprotein fractions after re-incubation with 40 mM-fucose, with limits of variability (lower pair of broken lines) around the mean ratio (large closed circle). Since the ratio of $^3\text{H}/^{14}\text{C}$ decreased after incubation with 40 mM-fucose for all glycoprotein fractions, tunicamycin inhibits the degradation of all fucosylated plasma-membrane glycoproteins in N18 cells. Because the range of $^3\text{H}/^{14}\text{C}$ ratios was much higher than the limits of variability for the data, it appeared that individual N18-cell membrane glycoproteins vary in their degree of resistance to degradation after glycosylation is inhibited. For example, three glycoprotein fractions with mol.wts. 118 000, 108 000 and 91 000 appeared to be less resistant to degradation after tunicamycin treatment than most of the cell-surface glycoproteins. The larger two of these fractions turn over rapidly in glycosylating N18 cells, whereas the smallest fraction turns over slowly (compare Figs. 3 and 4). Thus the degree of inhibition of degradation mediated by tunicamycin does not appear to correlate with the normal rate of turnover of these N18-cell membrane glycoproteins when tunicamycin is absent.

Discussion

Our data suggest that the presence of glucosamine or tunicamycin can inhibit the turnover of fucose of neuroblastoma-cell-membrane glycoproteins that were synthesized before addition of the inhibitors. Glucosamine acts by depleting UTP pools by formation of UDP-glucosamine and, thereby, inhibits the formation of other UDP-sugars, which are precursors to oligosaccharide synthesis (Bekesi & Winzler, 1969; Bosmann, 1971). The inhibitory effect of glucosamine on protein synthesis is most likely a reflection of an inhibition of RNA synthesis by the loss of available UTP. Tunicamycin inhibits lipid-carrier-mediated oligosaccharide synthesis (Tkacz & Lampen, 1975). In addition, tunicamycin preferentially inhibits glycosylation with relatively little effect on protein synthesis, whereas glucosamine inhibits both to about the same extent. The ability of tunicamycin to inhibit fucose turnover under nearly normal rates of protein synthesis argues for the inhibition of glycoprotein degradation being a result of the inhibition of oligosaccharide synthesis. Since inhibition of glycosylation (as shown by tunicamycin) or protein synthesis (Hudson & Johnson, 1977a) can affect the rates of fucose turnover, the effect of glucosamine on the degradation of previously synthesized glycoproteins probably results from a general inhibition of protein and glycoprotein synthesis. Because glucosamine

does not preferentially inhibit glycosylation, experiments utilizing glucosamine as a glycosylation inhibitor must be interpreted with caution.

Incubation with 0.5–1.0 μg of tunicamycin/ml for 4–5 h is commonly used to inhibit glycosylation in a wide variety of cultured cells (Duksin & Bornstein, 1977; Hickman *et al.*, 1977; Leavitt *et al.*, 1977; Olden *et al.*, 1978). However, 1 μg of tunicamycin/ml had no effect on glycosylation in N18 cells, although we found that fucose incorporation was inhibited over 90% in baby-hamster-kidney cells treated with as little as 0.5 μg /ml (results not shown). It appears, therefore, that N18 cells, in comparison with several other mammalian cell lines, are relatively resistant to the inhibitory action of tunicamycin.

Given that a link can be demonstrated between synthesis of the oligosaccharide portion of glycoproteins and fucose turnover, it should be possible to construct a mechanism for this phenomenon. Three models to explain the mechanism by which complete glycoproteins already in the plasma membrane might undergo decreased turnover on addition of a glycosylation inhibitor can be suggested. (1) The enzymes that degrade plasma-membrane glycoproteins are themselves short-lived glycoproteins. The unglycosylated enzymes produced in the presence of a glycosylation inhibitor would either be inactive or fail to be inserted into that environment, the lysosome or the plasma membrane, in which plasma-membrane glycoproteins are degraded. This model predicts that newly synthesized glycoproteins lacking oligosaccharide should also degrade more slowly. Protein-synthesis inhibitors also would decrease the activities of these enzymes and thereby inhibit degradation (Hudson & Johnson, 1977a). (2) Non-glycosylated glycoproteins might be more sensitive to degradation than their glycosylated counterparts. If so, then on addition of a glycosylation inhibitor, newly synthesized non-glycosylated glycoproteins would provide better substrates for proteolytic enzymes and would be preferentially degraded. Glycosylated proteins already present in the plasma membrane before addition of the inhibitor would thus degrade more slowly. If this model were correct, one would predict that glycoproteins synthesized in the presence of tunicamycin would degrade more rapidly; this has been shown to occur for the LETS glycoprotein in chick embryo fibroblasts (Olden *et al.*, 1978). (3) Inhibition of glycosylation or protein synthesis may alter glycoprotein degradation through the action of some regulatory molecule. One candidate for this role is cyclic AMP, since, in a previous study, we have shown that increased cyclic AMP concentrations in mouse neuroblastoma cells were shown to inhibit degradation of fucosylated plasma-membrane glycoproteins (Milenkovic *et al.*, 1978). However, several pieces of evidence rule out a relationship

between glycosylation inhibition by tunicamycin and increased cyclic AMP concentrations in neuroblastoma cells. First, tunicamycin treatment decreases the turnover of all fucosylated glycoproteins (Fig. 4), whereas cyclic AMP preferentially decreases the turnover of a specific population of high-molecular-weight rapidly metabolized membrane glycoproteins (Milenkovic *et al.*, 1978). Secondly, N18 cells exhibit no alteration in intracellular cyclic AMP concentrations after tunicamycin treatment. Thirdly, cyclic AMP does not inhibit glycosylation in neuroblastoma cells as measured by fucose incorporation (Milenkovic *et al.*, 1978).

Modulation in the glycosylation of glycoproteins may represent a regulatory mechanism in eukaryotic cells, especially to control cell-surface changes during the cell cycle or differentiation. By altering the oligosaccharide portions of cell-surface molecules involved in membrane transport, cell morphology and cell adhesion, hormone binding or density-dependent growth inhibition, a cell could modulate the appearance of these components at the plasma membrane or their degradation from the plasma membrane culminating in altered function. Changes in the glycosylation of degradation enzymes may inhibit their activity or their migration to the cell surface or the lysosomal compartment, providing another mechanism by which the degradation of surface components might be controlled.

Malignantly transformed cells exhibit alterations in the activities of glycosyltransferases (Warren *et al.*, 1973; Brady & Fishman, 1974), the degree of glycosylation of cell-surface components (Bosmann, 1972; Warren *et al.*, 1978) and plasma-membrane glycoprotein metabolism (Wallach, 1975; Parry, 1978). Changes in the normal pattern of glycosylation may be one cause of abnormalities in membrane transport, cell adhesion and morphology, and control of cell growth.

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