CELL SURFACE GLYCOPROTEINS I: ACCUMULATION OF A GLYCOPROTEIN ON THE OUTER SURFACE OF MOUSE LS CELLS DURING MITOSIS

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Surface glycopeptides derived from vertebrate cells have been separated into 4 classes by chromatography on DEAE cellulose columns. Among different cell types tested, significant differences were observed in the relative amounts of these 4 glycopeptide classes present on the cell surface. This type of heterogeneity is consistent with the expected biological role of cell surface glycoproteins. One glycopeptide, as revealed by the DEAE column analysis, was found to have a characteristic metabolic pattern in mouse LS cells. New accumulation of this structure, called glycopeptide 4, on the cell surface was detected only around the period of cell division (M phase) and not at other times during the cell cycle.

1. INTRODUCTION

In animal cells, a portion of the total cellular glycoprotein is found associated with the outer surface of the plasma membrane (1). Such cell surface glycoprotein components are now thought to be important in specifying cell recognition processes and as cell surface antigenic determinants. For example, a glycoprotein carries the M and N blood group specificities found on human red blood cells (2).

I have examined the properties of certain cell surface glycoproteins with a view toward establishing their role in cellular differentiation and development. Here I first describe the basic methods used for identifying surface glycopeptides and for separating them into 4 classes by chromatography on columns of DEAE cellulose. Different cell types examined in this way were found to contain quite different relative amounts of the 4 glycopeptide classes. Thereafter, I show how these methods have been used in conjunction with synchronously dividing cultures of mouse LS cells to clarify details of the cellular schedule for assembly of glycoproteins into the outer membrane. One glycopeptide studied in this way was found to accumulate on the cell surface preferentially at the time of cell division (M phase) and not at other times during the cell cycle.

2. METHODS

(a) *Cell growth*

All cells were grown in a gassed CO_2 incubator containing 95% air and 5%

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CO_2 using Falcon or NUNC plastic tissue culture plates. Except for cultures of L and LS cells, plates were collagenized by a standard method (3). Cell counting was done with a hemocytometer. Mouse LS cells adapted for growth in suspension culture were the kind gift of Dr. A. Newton. They were grown in Eagle's Minimal Essential Medium (MEM) plus 10% calf serum, penicillin and streptomycin.

Primary cultures of chick embryo myoblasts were prepared from 10 day embryos by the method of Bischoff and Holtzer (4) and grown in 8 parts MEM, 1 part horse serum and 1 part chick embryo extract. These cultures were maintained for 3 days during which separated myoblast cells fused with each other to form a network of myotubes as expected. Cell density and [^{3}H]glucosamine labeling conditions are given in the legend to Fig. 1.

Primary cultures of chick spinal cord cells were prepared from 6 day chick embryos; sympathetic ganglion cells were from 10 day embryos. The appropriate tissue was dissected out of the embryo and trypsinized; approximately 6 x 10^5 cells were grown on a 50 mm plate in MEM plus 10% horse serum and antibiotics. In the case of sympathetic ganglion cells the medium was supplemented with glucose and 5 µg per ml of Nerve Growth Factor (the gift of Dr. D. Bray) to promote neurite outgrowth. Three mM hydroxyurea was added to cultures of spinal cord cells to inhibit growth of a small fraction (approximately 10%) of fibroblast cells which ordinarily contaminate these cultures. Nerve cell cultures were maintained for 2 to 3 days during which extensive outgrowth of dendrite-like processes was observed; neither of these types of nerve cells divides in culture.

(b) *Isolation of cell surface glycopeptides*

Cellular glycoproteins were labeled preferentially by growing cells in the presence of [^{3}H]glucosamine. In agreement with previous studies of this type (5-6), glucosamine was found to be incorporated into glycoprotein as amino sugar or sialic acid; it is not metabolized to other cellular precursors such as neutral sugar (less than 10%) or amino acid (undetectable amount). Labeled glycopeptides derived from cell surface, as opposed to internal glycoproteins, were obtained by trypsinization of the cells. After growth in [^{3}H]glucosamine cells were washed in Earle's Balanced Salt Solution (BSS) and then exposed to 0.25% trypsin (Flow Laboratories) in BSS for 30 min at room temperature with gentle agitation. (These conditions closely resemble the method ordinarily used for routine subculturing of cells.) Since cells survive this procedure and are neither lysed nor killed, one reasons that the resulting soluble glycopeptides were derived mainly from the cell surface. This reaction is absolutely dependent on the presence of trypsin and proceeds, with the cells studied here, at a rate comparable to the rate observed for solubilization of a glycopeptide derived from human red blood cells (7).

Glycopeptides solubilized in this way were separated from each other by chromatography on a 1 x 15 cm column of Whatman DE52 DEAE cellulose. The column was eluted with a 500 ml gradient of 0.01 M to 0.15 M NaCl in 2 x 10^{-3} M Tris·Cl buffer of pH 7.5. Five ml fractions were collected and a 1 ml aliquot of each was counted in Bray's solution (8). Glycopeptides not bound to DEAE cellulose were not isolated by this procedure; no glycopeptide material was found to remain on the column after elution with 0.15 M NaCl.

(c) *Selection of synchronously dividing mouse LS cells*

Synchronized populations of mouse LS cells were obtained by the method of Shall and McClelland (9). A column of medium was prepared in a 50 ml graduated cylinder and warmed to 37°. Three ml of asynchronous cells at a density of approximately 2 x 10^7 cells per ml were then carefully layered on the top of the column and the column incubated for 45 min at 37°. Cells from the top 4 ml were harvested and found to be in early G1 phase. The generation time

for such cultures was found, quite reproducibly, to be 24 hr.

3. RESULTS

(a) *Analysis of cell surface glycoproteins*

Different cell types were labeled with radioactive glucosamine in culture. In each case the glycoproteins on their surfaces were sampled by trypsinizing the cells; this procedure releases labeled glycopeptides from the cells. It is assumed that these released glycopeptides are derived solely from plasma membrane, and not from intracellular, glycoproteins. Under the trypsinization conditions employed here, a substantial fraction of the total cellular glucosamine labeled material was solubilized. In the case of mouse L cells, for instance, this amounted to some 87% of the total glucosamine incorporated into the cells. More prolonged trypsin treatment was not found to result in more soluble glycopeptide, nor did it affect the spectrum of glycopeptides produced as described below. Accordingly, one reasons that the glycopeptides obtained by this procedure are the result of complete, not partial, trypsinization.

Glycopeptides harvested in this way were separated from each other by chromatography on DEAE cellulose columns. Thus, glycopeptides carrying a net negative charge (due presumably to the presence of terminal sialic acid residues on the carbohydrate portions), but not neutral and positively charged glycopeptides, are resolved by this method. The proportion of the total cellular glucosamine labeled material present in the negatively charged fraction was found to be different for different cell types tested, but never less than approximately 30%. In the case of L cells 50% of the total cellular glucosamine label was analyzed on the DEAE cellulose column as shown in Table 1.

Glycopeptides derived from 10 different cell types have now been analyzed in this way and a representative sample of the results is shown in Fig. 1. In general, glycopeptides from a single cell type could be classified into 4 groups of glycopeptides which elute from the DEAE cellulose column at approximately 0.03 M, 0.04 M, 0.06 M and 0.07 M NaCl; for convenience, these are called glycopeptides 1, 2, 3 and 4 respectively. It is clear, however, that one cannot regard glycopeptides 1, 2, 3 and 4 as single, homogeneous molecular components; all could be mixtures of similar glycopeptides.

Different cell types were found to contain markedly different amounts of these 4 glycopeptide groups. Thus, for example, in chick spinal cord cells glycopeptide 4 is most prominent whereas with myoblasts the yield of glycopeptide 2 is greatest. Although most cell types had at least a small amount of all 4 glycopeptides, there are exceptions to this rule. For instance, mouse L cells are lacking glycopeptide 3 as shown in Fig. 1. No cell type tested was found to contain more than the usual 4 glycopeptides.

All 4 glycopeptides from chick sympathetic ganglion cells were subjected to further analysis by chromatography on Sephadex G-25 columns. All were found to be excluded from these columns and therefore cannot be regarded, for instance, as low molecular weight intermediates in the synthesis of something else.

(b) *Glycoprotein appearance during mitosis*

The cellular schedule for assembly of glycoproteins into the outer membrane was examined by applying the DEAE cellulose method of analysis to synchronously dividing populations of mouse LS cells. These cells, which have a spherical morphology, were derived from normal fibroblast-shaped L cells by adapting them for growth in suspension culture. The glycopeptide patterns, as described above, for L and LS cell lines were found to be identical.

Beginning in G1 phase, such synchronously dividing populations of LS cells were cultured for 2 generations in the presence of [^{3}H]glucosamine.

TABLE 1

Distribution of glucosamine label in mouse L cells

Fraction	Glucosamine incorporated	
	CPM/10^6 cells	%
Total cells	103,350	100
Trypsin-sensitive and analyzed on DEAE column	51,850	50
Trypsin-sensitive but not analyzed on DEAE column	38,138	37
Trypsin-insensitive	13,362	13

A log phase culture of L cells was grown for 48 hr in a 50 mm plastic tissue culture plate containing 4 ml of medium and 50 μCi of [^{3}H]glucosamine (2.6 Ci per mmole). The final number of cells was 1.8 x 10^6. Cells were washed, scraped off the surface of the plate and suspended in BSS. The total [^{3}H]glucosamine label incorporated into acid precipitable material was determined before and after trypsin treatment by precipitating an aliquot of cells with 5% trichloroacetic acid (TCA) at 0°. Precipitates were filtered on Whatman GF/C glass fiber filters, washed with cold 5% TCA, dried and counted in a standard liquid scintillation medium. Cells were trypsinized and the solubilized glycopeptides analyzed by chromatography on DEAE cellulose columns as described above. All glycopeptide material bound to the column and not eluted with 2 x 10^{-3} M Tris·Cl buffer of pH 7.5 was included in this analysis; trypsin sensitive glycopeptides not detected in this way were assumed not to have been bound to the DEAE cellulose column. Results are corrected to a common counting efficiency of 5% and expressed as counts per minute per 10^6 cells.

Aliquots of cells were harvested at various times and the amount of glycopeptide 4 present was determined by trypsinization followed by DEAE cellulose chromatography as described above. Qualitatively, the profiles obtained were similar to that shown in Fig. 1 for L cells; no glycopeptides other than glycopeptides 1, 2 and 4 were observed at any time during the cell cycle. However, as shown in Fig. 2, quantitative appearance of glycopeptide 4 was found to be closely correlated with the period of cell mitosis and quite distinct from the interval of DNA synthesis (S phase) which occurs some 6 hr earlier. No such restricted period was observed for accumulation of glycopeptides 1 and 2 on the cell surface; they appeared at a relatively constant rate throughout the cell cycle.

In contrast to the situation with synchronously dividing cultures as shown here, asynchronous cultures of LS cells incorporate [^{3}H]glucosamine into glycopeptide 4 at a constant rate beginning no later than 5 hr after the isotope is added. Thus, it is unlikely that the lag in labeling of glycopeptide 4 observed in synchronous cultures is due to equilibration of added [^{3}H]glucosamine with a pool of non-radioactive precursors.

Once glycopeptide 4 has appeared in trypsin-sensitive form on the cell surface, it does not later become masked, degraded or otherwise unavailable

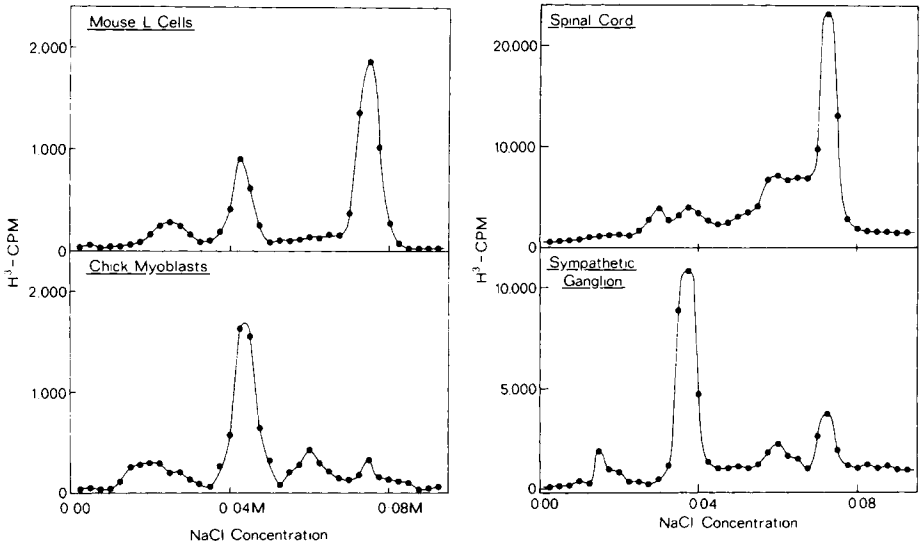

Fig. 1. *Glycopeptide species derived from different cell types by trypsinization.* Cell cultures were prepared as described above, grown in 50 mm culture plates and exposed to 100 μCi [^{3}H]glucosamine (2.6 Ci per mmole) in a total of 4 ml of medium for 48 hr. Mouse L cells were labeled during log phase of growth. Chick myoblast cells were exposed to [^{3}H]glucosamine during the interval of 24 to 72 hr after establishment of the culture when the rate of cell fusion was highest. Nerve cells were labeled beginning 12 hr after culturing. Final cell numbers analyzed were: L cells, 5 x 10^6 cells; myoblasts, 3 x 10^7 cells; chick spinal cord and sympathetic ganglion, 6 x 10^5 cells each. Cells were trypsinized and the resulting soluble glycopeptides analyzed by DEAE cellulose chromatography as described in Methods. Glycopeptides 1, 2, 3 and 4 are eluted at 0.03 M, 0.04 M, 0.06 M and 0.07 M NaCl respectively.

to enzymatic digestion. Thus, the amount of glycopeptide 4 detectable on the cell surface does not decrease after M phase is complete as shown in Fig. 2. This contrasts to the behavior, for instance, of the H-2 histocompatibility antigens in P815Y cells which become alternately less (during S phase) and more (during G2, M and G1) available for binding to antibodies directed against their determinant groups (10).

4. DISCUSSION

No biochemical study has yet established an upper limit to the relative diversity of glycoprotein species present on the outer surface of a dividing animal cell type. The large number of antigenic specificities characteristic of cell surfaces leads one to suspect that the number of different glycoproteins may be correspondingly large. On the other hand, studies on human erythrocytes, which have only 1 major glycoprotein component, suggest that a rather simpler pattern may be the general rule. The results presented here are more consistent with this second, simpler view of glycoprotein heterogeneity. Glycopeptides derived specifically from cell surface glycoproteins could be divided neatly into only 4 classes by chromatography on DEAE cellulose columns. One must appreciate, in this regard, that not all (but still a substantial fraction) of the cell surface glycopeptides are included in

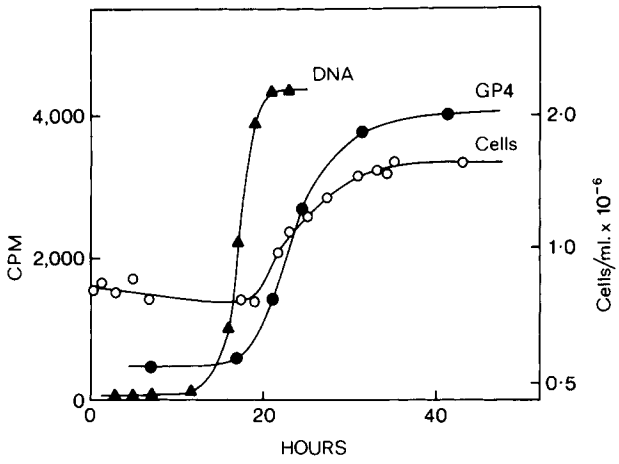

Fig. 2. *Appearance of glycopeptide 4 (GP4) on the cell surface during mitosis.* A synchronously dividing population of 7 x 10^6 LS cells was obtained as described above and cultured at 37° in 9 ml of medium containing 250 µCi [^{3}H]glucosamine and 20 µCi [^{14}C]thymidine (45 mCi per mmole). Separate aliquots were taken to measure DNA synthesis (▲), glycopeptide 4 appearance (●) and cells per ml (○). Incorporation of [^{14}C]thymidine into acid insoluble material was determined as a measure of net DNA synthesis. One-tenth ml aliquots of the culture were precipitated with 5% trichloroacetic acid, which lyses the cells, and filtered on Whatman GF/C glass fiber filters. Washed filters were counted in a Nuclear-Chicago liquid scintillation system adjusted to count [^{14}C] but not [^{3}H]. Less than 0.1% of the [^{14}C] counts recorded could have been due to contaminating [^{3}H]. One ml aliquots of the culture were taken for glycopeptide analysis. Cells were washed with BSS and trypsinized as usual. The total number of [^{3}H] counts present as glycopeptide 4 was determined after separation of glycopeptides by DEAE cellulose chromatography as described above. Only [^{3}H]labeled, no [^{14}C]labeled, material was found in this fraction.

this analysis, that glycopeptides 1, 2, 3 and 4 may actually be mixtures of glycopeptides, and that minor glycopeptides present in only a few copies per cell may not have been observed by the methods used here. Nevertheless, the spectrum of glycopeptides obtained from individual cell types is much less complex than one would have expected from a very heterogeneous population of cell surface glycoproteins. One is led to suspect, therefore, that the number of distinct surface glycoprotein species is fairly small.

In contrast to the situation with a single cell type, which may have only a few distinct species of glycoprotein, one expects that among different cell types quite a wide variety of glycoproteins will be observed. For instance, if cell surface glycoproteins are involved in specific cell-cell recognition processes during development, then different cell types ought to have quite characteristic and individual sets of surface glycoproteins. The results presented here are not incompatible with this expectation. Different cell types tested were found to contain quite significantly different relative amounts of the 4 glycopeptide classes separated by DEAE cellulose chromatography. Furthermore, since the same glycopeptide class derived from 2 different cell types need not be the same, more detailed chemical studies of

these glycopeptides may reveal other levels of heterogeneity that could be of biological significance. In addition to qualitative differences among glycopeptide species, it is also highly likely that different cell types will show quantitative differences in the total amount of glycoprotein present on the surface. For instance, Fig. 1 shows that the total yield of glycopeptide from 6×10^5 spinal cord cells is relatively greater than that from 5×10^6 L cells. It seems unlikely that this difference is entirely due to differences in cell size or in specific radioactivity of the glycopeptides. More probably, glycoproteins on the surface of spinal cord cells are either more densely packed or richer in carbohydrate than those of L cells.

Experiments with synchronously dividing populations of mouse LS cells were undertaken to answer basic questions about the metabolic program for assembly of glycoproteins into the cell surface. Appearance of a glycopeptide in trypsin-sensitive form on the cell surface is taken as evidence that its parent glycoprotein has been physically assembled onto the outer membrane. It should be emphasized that this method of analysis detects assembly of glycoproteins onto the cell surface and not glycoprotein biosynthesis.

In general, one expects that, compared to other parts of the cell cycle, the period of cell division will be characterized by much assembly of new membrane and other cell surface structures. For example, a 25% increase in total surface area is expected when a spherical cell (such as the LS cell) divides provided that the total volume remains constant during the time of division. Preferential appearance of glycopeptide 4 on the cell surface during M phase, as shown here, is quite compatible with this expectation. One reasons that the parent glycoprotein from which glycopeptide 4 was derived is itself assembled onto the cell surface during M phase and not at other times during the cell cycle. In this context it would be of interest to know whether all or only a subset (including the glycopeptide 4 parent glycoprotein) of all membrane components are assembled onto the cell surface at the time of mitosis. It is possible, for example, that only those structures physically involved in separation of the 2 daughter cells are assembled onto the outer surface during cell division. Synchronous cultures of LS cells, as employed here, would appear to be an adequate experimental system for such an investigation.

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