Endoglycosidase H-sensitive glycopeptides in eleven different animal cells

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We have examined the distribution of mannose-labelled glycopeptides in eleven different animal cells grown *in vitro*. All of the cells examined contained endoglycosidase H-sensitive species of high and low molecular weight, associated with the cell material and with the cell surface; however, the distribution between the two pools was different, suggesting a 'sorting out' of glycoproteins. Another conclusion from our studies is that the oligosaccharide processing known to occur during or after membrane glycoprotein translation is incomplete in a high percentage of mannose-containing *N*-linked oligosaccharides of cell surfaces. There was no consistent correlation between the relative amounts of endoglycosidase H-sensitive and -resistant glycopeptides and whether the cells were normal, virus-transformed or tumour-derived.

It is generally assumed that the surface of mammalian cells contain two classes of asparagine-linked glycopeptides. Glycopeptides labelled with a radioactive sugar precursor can be released from cells by mild proteolytic treatment. By this method, approx. 10–20% of the radioactivity can be recovered, and we have defined this fraction as 'cell-surface material'; the remaining radioactivity has been referred to as 'cell material' (Muramatsu *et al.*, 1973). The labelled glycopeptides, whether surface or cell, can be operationally separated into 'acidic' and 'neutral' by high-voltage paper electrophoresis (Ceccarini *et al.*, 1975) or by concanavalin A affinity chromatography (Blithe *et al.*, 1980). The acidic species terminate with sialic acids and have the following average sugar composition (residues/molecule): *N*-acetylglucosamine, 3–5; mannose, 3; galactose, 1–3 and sialic acid 1–3 (Atkinson & Hakimi, 1980). The neutral species terminate mainly with neutral sugars, with the most abundant monosaccharide being mannose (Atkinson & Hakimi, 1980; Tai *et al.*, 1975). Although numerous studies have established both growth-dependent and transformation-dependent alterations in these two classes of glycopeptides [see Warren *et al.* (1978) for review], no systematic study has been undertaken to establish the generality of these two classes of glycopeptides.

Specific glycosidases can be used to study the carbohydrate composition of glycopeptides (Muramatsu *et al.*, 1976). One such enzyme, endoglycosidase H (Tarentino & Maley, 1974), is

capable in general of hydrolysing the chitobiose linkage of the neutral glycopeptides, but does not hydrolyse the same linkage in the acidic species. Thus a mixture of acidic and neutral species will yield digested and undigested products which can be distinguished by gel-filtration chromatography. In the present paper we have investigated the ratio between acidic and neutral glycopeptides from eleven different cells.

Materials and methods

For these studies we grew five human cell types, two of which are tumour-derived (HeLa and KB) and three are normal fibroblasts (KL2, WI38 and IMR-90), and normal secondary chick-embryo fibroblasts. We also studied the cell lines CHO and BHK (hamster), 3T3 (mouse), L-cells (mouse) and VERO (monkey).

The growth conditions for all of the established cells were reported previously (Muramatsu *et al.*, 1973), except that secondary chick fibroblasts were prepared as described by Hakimi & Atkinson (1980). In all cases the experimental cultures were plated in large glass vessels (Blake bottles) and fed daily with Eagle's minimal essential medium buffered with organic buffers at the optimum pH (Ceccarini, 1975). When approx. 50–75% of the growing area was covered with rapidly growing cells, the radioactive precursor was added. The radioactive sugar precursors which we used were [³H]-mannose (sp. radioactivity 13.8 Ci/mmol) and [¹⁴C]-mannose (sp. radioactivity 216.5 mCi/mmol). We have previously demonstrated that, in mammalian

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cells, radioactive mannose was converted only into fucose and hexosamine (10% or less) when the cells were labelled for 20–24 h (Ceccarini & Atkinson, 1977). The labelling of each cell was done in pairs; that is, one Blake bottle received [^3H]mannose (5 $\mu\text{Ci}/\text{ml}$) while the other flask was labelled with [^{14}C]mannose (0.5 $\mu\text{Ci}/\text{ml}$). After 24 h the incorporation was terminated and the cells were treated with Pronase as previously described (Muramatsu *et al.*, 1973). From each flask two different preparations were obtained, which we term 'cell-surface' and 'cell-remainder' material. Cell-surface material could be removed by mild non-disruptive proteolysis of intact cells; cell-remainder material was not so removed. The cell-surface fraction labelled with [^3H]mannose from one flask was mixed with the cell-remainder fractions, [^{14}C]mannose-labelled, from the other flask, and the mixture was digested exhaustively with Pronase (Muramatsu *et al.*, 1973). The Pronase was heat-inactivated and the mixture fractionated through a Sephadex G-50 (fine grade) column. The glycopeptide fraction was pooled, freeze-dried and digested with endoglycosidase H as previously described (Muramatsu *et al.*, 1976). The volume of the final incubation mixture ranged from 0.2 to 0.4 ml and the digestion was carried out for 24 h at 37°C. Longer incubation periods or more enzyme did not release more oligosaccharides. The digested sample was boiled for 10 min and the insoluble material was removed by centrifugation. The mannose-labelled glycopeptides were separated into endoglycosidase H-resistant and -susceptible oligosaccharides by use of Bio-Gel P2 columns [0.9 cm $\times$ (145–170) cm]. These columns were equilibrated and eluted with water containing 1 mM- NaN_3 or with 0.5 M-acetic acid. Radioactivity in fractions was determined in a liquid-scintillation counter. High-resolution Bio-Gel P4 column chromatography was performed as described previously (Hakimi & Atkinson, 1980); columns were equilibrated and eluted with 0.1 M-ammonium acetate. Marker oligosaccharides (200 μg per column) were detected by using the phenol/ H_2SO_4 assay (Dubois *et al.*, 1956) and consisted of oligo-C3 ($\text{Man}_3\text{GlcNAc}_3$), oligo-D3 ($\text{Man}_6\text{GlcNAc}$) and oligo-E3 ($\text{Man}_3\text{GlcNAc}$) (see Carver *et al.*, 1981), stachyose, raffinose, sucrose and mannose; bovine serum albumin marked the excluded volume.

Results and discussion

The use of the same sugar precursor, mannose, but labelled with two different isotopes (^3H and ^{14}C), permitted us to compare directly the susceptibility of the cell-surface and cell-remainder glycopeptides to endoglycosidase H. Cells used in the present study were in the exponential phase of their growth curve, in which we had previously observed that oligo-

mannosyl species were particularly enriched (Ceccarini *et al.*, 1975; Muramatsu *et al.*, 1976).

The mannose-labelled cell-surface and cell-remainder material was initially partially purified and the glycopeptides digested with endoglycosidase H as described in the Materials and methods section. The glycopeptides were fractionated by Bio-Gel P2 column chromatography. Glycopeptides not digested always eluted near the void volume of the column, whereas digested species were retarded. Two typical fractionations (shown in Figs. 1a and 2)

Fig. 1. Fractionation of endoglycosidase H-digested glycopeptides by column chromatography (a) Bio-Gel P2 (–400 mesh) chromatography of mannose-labelled glycopeptides from VERO cells: ○, cell-surface glycopeptides; ●, cell-remainder glycopeptides. The arrows, left to right, represent the following markers: bovine serum albumin, $\text{Man}_3\text{GlcNAc}_3$, stachyose, raffinose, sucrose and mannose. (b) Bio-Gel P4 (–400 mesh) chromatography of oligosaccharides released by endoglycosidase H (obtained from a, fractions 92–130). The column measured 0.9 cm $\times$ 170 cm and fractions (0.5 ml) were collected as previously described (Hakimi & Atkinson, 1980). The arrows left to right represent the following markers: bovine serum albumin, $\text{Man}_3\text{GlcNAc}_3$, $\text{Man}_6\text{GlcNAc}$, $\text{Man}_3\text{GlcNAc}$, stachyose and raffinose. (c) Mannose-labelled oligosaccharides from chick fibroblasts. The markers and the column conditions were identical with those in (b).

Fig. 2. Fractionation of endoglycosidase H-digested glycopeptides by Bio-Gel P2 (—400 mesh) chromatography HeLa, S3: O, cell-surface glycopeptides; ●, cell-remainder glycopeptides. The column measured 0.9 cm × 145 cm, was eluted with water plus 1 mM-Na₂SO₄, and 0.6 ml fractions were collected and analysed for radioactivity. The arrows, left to right, represent the following markers: bovine serum albumin, stachyose, raffinose, sucrose and mannose. Inset: [¹⁴C]mannose-labelled endoglycosidase H-sensitive material was digested with jack-bean α -mannosidase and re-chromatographed on Bio-Gel P2. The elution was carried out with 1.0M-acetic acid. The arrows, left to right, indicate stachyose, raffinose, sucrose and mannose.

demonstrate that a portion of the radioactivity was eluted near the void-volume region and another fractionated in the 1800–800 mol.wt. region. This region is clearly heterogeneous, probably ranging in composition from three to eight mannose residues (Muramatsu *et al.*, 1976). This conclusion was further reinforced when the digestion products of VERO and chick fibroblasts were further fractionated on Bio-Gel P4 (—400 mesh). This fractionation appeared to separate the products into unique species which co-migrated with authentic markers, namely GlcNAc₃Man₃, Man₆GlcNAc and Man₅GlcNAc. It has been shown that oligosaccharides containing a higher proportion of *N*-acetylglucosamine to mannose chromatograph as if they have a higher molecular weight (Hakimi & Atkinson, 1980). However, these unique species could also represent oligomannosyl species containing as many as eight and nine residues. In fact, digestion of the endoglycosidase H-released oligosaccharides from HeLa and KL2 cells with α -mannosidase supports this conclusion (see below). The high degree of resolution of the Bio-Gel P4 also permits us to conclude that these species were quantitatively

different in the cells studied and that cellular and surface components were readily distinguishable.

A comparison between the digested and undigested glycopeptides was made in eleven cells, and the results are presented in Table 1. Our data demonstrate that all of the cells studied contained a certain fraction of glycopeptides that could be hydrolysed by endoglycosidase H. In some cells, like VERO and WI38, the degree of hydrolysis was similar in both the cell-surface and cell-remainder material. On the other hand, in other cells, like IMR-90, BHK and 3T3, the cell-surface glycopeptides were relatively resistant to digestion with endoglycosidase H (range 68–79%), whereas the cell-remainder glycopeptides appeared particularly enriched in neutral species (range 46–50%). In general, however, no clear correlation could be made between the origin of the eleven different cell types used in the present study and the overall distribution of the glycopeptides.

We had previously demonstrated that acidic glycopeptides from animal cells in tissue culture were not digested by endoglycosidase H (Muramatsu *et al.*, 1976), whereas they were

Table 1. Analysis of endoglycosidase H-sensitive and -resistant glycopeptides in eleven different cells, grown *in vitro* during the exponential phase of growth

Cell line	Percentage of total glycopeptides			
	Endoglycosidase-sensitive		Endoglycosidase-resistant	
	Surface	Cell	Surface	Cell
VERO	34.6	39.3	65.4	60.7
CHO	56.7	36.3	43.3	63.7
L	42.7	60.6	57.1	39.4
IMR-90	31.6	49.9	68.4	50.1
BHK	31.9	46.4	68.1	53.6
WI38	41.9	43.8	58.1 (99.5)*	56.2 (100)
KL2	45.6	57.2†	54.4 (98.9)	42.8 (97)
3T3	21.1	46.3	8.9	53.7
HeLa	45.2	54.5‡	54.8	45.5
KB	34.7	51.8	65.3	48.2
CEF	46.0	51.6	54.0	48.4

* Percentage of radioactivity not released by α -mannosidase.

† Upon digestion with α -mannosidase, 64% of the radioactivity was released as free mannose, 9% as Man β 1 $\rightarrow$ 4GlcNAc, whereas the remaining material (27%) appeared to be similar in molecular weight to the endoglycosidase H digestion products. In another cell line (WI26Va), not included in the above list, 68, 6 and 26% respectively of the mannose radioactivity was similarly accounted for.

‡ The results of the α -mannosidase digestion are shown in the inset of Fig. 2.

converted into oligosaccharides when digested with endoglycosidase D in the presence of exoglycosidases (Muramatsu *et al.*, 1973, 1976; Ceccarini & Atkinson, 1977). However, it is not known whether endoglycosidase H can hydrolyse all known hybrid-type glycopeptides (e.g. ovomucoid; Yamashita *et al.*, 1982). Since our endoglycosidase H preparation, when tested, lacked exoglycosidases, we could thus infer that the undigested species must be acidic or hybrid species. This hypothesis was directly tested by the following analyses. When undigested mannose-labelled material from WI38 and KL2 cells (cellular and surface) was treated with jack-bean-meal α -mannosidase, 97% or more of the radioactivity eluted from Bio-Gel P2 columns as still intact glycopeptides (Table 1). The remaining radioactivity chromatographed with authentic mannose. Similar results were obtained with two other cells, WI8Va and WI26Va (results not shown). Thus the undigested, unlike the digested, material (see below), must contain little if any terminal mannose and thus our hypothesis would appear correct. As an example of the presence of resistant hybrid species, one of us (P. H. Atkinson, un-

published work) showed that certain mannose-labelled chick glycopeptides, when digested in different orders with *N*-acetylneuraminidase, β -galactosidase and exo-*N*-acetylglucosaminidase, became susceptible to endoglycosidase H digestion only when galactose was removed. Furthermore, these glycopeptides could also be converted into oligosaccharides when digested with endoglycosidase D, an enzyme that fails to cleave the chitobiose linkage when six or more mannose residues are present (Muramatsu, 1978).

The endoglycosidase H-digested cellular products from two representative cell types, KL2 and HeLa, were also digested with the α -mannosidase and subsequently analysed by column chromatography. In the case of HeLa cells, only two products were released, one which co-migrated with authentic mannose and the other corresponded to a substance having an apparent mol.wt. of 530 (Fig. 2, inset). ^1H n.m.r. analysis of a substance obtained similarly from ovalbumin glycopeptide D (Carver *et al.*, 1981) showed it to be Man β 1 $\rightarrow$ 4GlcNAc. The results obtained with KL2 revealed, in addition to the free sugar and the disaccharide, material that had an apparent molecular weight similar to the products of the endoglycosidase H digestion (Table 1). Similar results (not shown) were also obtained with the WI26Va cell. These results are consistent with the presence of mannose-containing hybrid-type oligosaccharides that are digested by endoglycosidase H but could have terminal α -mannosidase-insensitive residues. Selected removal of terminal α -linked mannose residues has been reported recently (Berman & Allerhand, 1981; Byrd *et al.*, 1982).

The arbitrary method we have used to distinguish between cell-surface and cell-remainder appears valid, since in all cases the cells are not damaged and the two fractions can be easily recognized. We argue, therefore, that the cell-surface fraction contains mainly those glycopeptides which are accessible to mild proteolytic digestion, whereas the cell-remainder fractions contain those species which are deeply embedded in the surface matrix or are associated with other cell organelles. Since it appears likely that glycoproteins are membrane-bound from the time of translation and transported via the endoplasmic reticulum and Golgi systems to the plasma membrane, it would seem reasonable to suggest that some sorting out must take place to account for the differential susceptibility of the different classes of glycopeptides to mild proteolysis. Evidence for the presence of distinct intracellular pathways for glycoprotein has been recently presented (Chapman *et al.*, 1979; Cammarata *et al.*, 1981; Gumbiner & Kelly, 1982). Another conclusion from our studies is that the oligosaccharide processing known to occur during or after mem-

brane-glycoprotein translation is incomplete in a large percentage of mannose-containing *N*-linked oligosaccharides of cell surfaces. The findings extend those of Burke & Keegstra (1979), who found high-mannose-containing substances in Sindbis-virus glycoproteins.

The degree of structural heterogeneity in these types of oligomannose and hybrid oligosaccharides has been well established [see Atkinson & Hakimi (1980) for a review]. However, the regulatory importance of these substances remains to be elucidated. The best example of structural heterogeneity of oligomannosyl glycopeptides has been demonstrated in chick ovalbumin (Tai *et al.*, 1975, 1977; Atkinson *et al.*, 1981). Their ubiquitous presence in the eleven animal cells studied here suggests that they must play an important role in cell regulation. In fact, recent data appears to implicate these classes of glucopeptides in cell-cell interactions in developing systems (Grabel *et al.*, 1979, 1981; Carson & Lennarz, 1981; Hart, 1982).

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