Growth-dependent alterations in oligomannosyl cores of glycopeptides

(cell surface/specific glycosidases/neutral acidic glycopeptides)

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ABSTRACT Mannose-labeled glycopeptides from the surface of growing and nongrowing human diploid cells (KL-2) were separated into neutral glycopeptides and acidic glycopeptides by paper electrophoresis. Growth-dependent alterations occurred in oligomannosyl cores of the neutral glycopeptides; namely, the neutral glycopeptides from the surface of growing cells were more resistant to endo-\(\beta\)-N-acetylglucosaminidase D but were more susceptible to \(\alpha\)-mannosidase (EC 3.2.1.24; \(\alpha\)-D-mannose mannohydrolase) than those derived from the surface of nongrowing cells. Another growth-dependent change was found when the endoglycosidase-resistant material from acidic glycopeptides was compared by paper electrophoresis at pH 1.9. The material from the surface of nongrowing cells contained a component that was absent or greatly reduced in growing cells.

Radioactive fucose has been conveniently used to detect growth-dependent pattern changes of cell surface glycopeptides of various cultured cells (1–4). However, distribution of fucose in glycoproteins is restricted to a certain class of carbohydrate chains (5). In order to study growth-dependent pattern changes of glycopeptides from a more general viewpoint, we used radioactive mannose as a precursor to label the glycopeptides, since mannose residues are universal in \(\beta\)-glycoside-type glycopeptides (5). We have used, in combination with single, four distinct methods of distinguishing different types of glycopeptides. These methods include (i) differential susceptibility to endo-\(\beta\)-N-acetylglucosaminidase D (6), which leads to information on the core region of glycopeptides; (ii) differential susceptibility to \(\alpha\)-mannosidase (EC 3.2.1.24; \(\alpha\)-D-mannose mannohydrolase), which leads to information on glycopeptides terminating in \(\alpha\)-mannosyl residues; (iii) electrophoresis of the endoglycosidase-resistant material, which leads to information on charge differences; and (iv) separation of glycopeptides into acidic and neutral species, which facilitates analysis by the above methods and by itself leads to information on differently sized glycopeptides.

We report that structural alterations dependent on cell growth status in cell surface glycopeptides seem ubiquitous throughout the oligosaccharide chains and may also involve the peptide moieties.

MATERIALS AND METHODS

Cells, Labeling, and Cell Surface Glycopeptides. Human diploid fibroblasts (KL-2) were cultured as described under conditions where the pH of the culture medium was maintained optimal for their growth (4). Cells were labeled with radioactive mannose for 24 hr, either in the growing state or in the nongrowing state (4). [2-\(^3\)H]Mannose (1.0 Ci/mmol, Amersham-Searle) was used at a concentration of 5 \(\mu\)Ci/ml of the culture medium and [\(^14\)C]mannose (45 mCi/mmol, New England Nuclear; 240 mCi/mmol, Schwartz-Bioresearch, Inc.) was used at a concentration of 0.05–0.5 \(\mu\)Ci/ml.

Cell surface material was released from the intact cells by brief Pronase digestion under conditions such that less than 0.5% of cells were lysed (4). Glycopeptides from cell surface material were prepared by extensive Pronase digestion and purified by Sephadex G-50 column chromatography (ref. 4 and Fig. 1A). The ratio of [\(^3\)H]mannose or [\(^14\)C]mannose-labeled cell surface glycopeptides to cell material glycopeptides was 1 to 9 in both the growing cells and nongrowing cells. The [\(^3\)H]mannose or [\(^14\)C]mannose we concluded to be incorporated into glycopeptides without significant conversion into other sugars, since virtually all the radioactivity in the hydrolysate of any glycopeptide preparations by 1 M HCl for 4 hr at 100°C was located in the mannose region after paper chromatography in butanol/pyridine/water (6:4:3). Furthermore, no conversion into sialic acids was detected because after hydrolysis of glycopeptides by 0.05 M H\(_2\)SO\(_4\) at 80°C for 1 hr and paper electrophoresis at pH 6.5, there was no radioactivity comigrating with these sugars. No significant conversion to fucose has been detected after hydrolysis at 100°C for 45 min by 0.1 M HCl, followed by paper chromatography.

Glycosidases. Preparation, specificity, and use of neuraminidase, \(\beta\)-galactosidase, and \(\beta\)-N-acetylglucosaminidase on various plasma-type glycopeptides, including those of KL-2 cells, has been described (4). The endoglycosidase active on these glycopeptides has also been described (4, 8) and its specificity fully defined (endo-\(\beta\)-N-acetylglucosaminidase D; ref. 6). \(\alpha\)-Mannosidase from jack bean meal was purified according to Snaith and LeVvy (7). The enzyme was free from other glycosidases and released no radioactive sugars from mouse myeloma IgG glycopeptide label and with [\(^3\)H]fucose; [\(^3\)H]galactose, or [\(^3\)H]glucosamine (8).

Preparative Electrophoresis. Glycopeptides, purified by Sephadex G-50 chromatography, were separated into acidic and neutral species by high voltage paper electrophoresis on 1-inch (2.5 cm) strips of Whatman 3MM paper at pH 6.5 (pyridine/acetic acid/water, 10:0.4:89.6), 35–40 V/cm for 3–4 hr. The paper was divided into 1-cm fractions (40 to the positive pole, 20 to the negative pole) and eluted 30 min at room temperature in 1.0 ml of water; aliquots were analyzed for radioactivity to determine the glycopeptide distribution (Fig. 1B). Acidic glycopeptides were those found from 2 to 30 cm to the positive side of the origin; neutral glycopeptides were those found from 4 to 12 cm to the negative side.
of the origin (Fig. 1B), the region where neutral sugars (i.e., galactose) migrated. These pools were lyophilized and resuspended in a small volume (0.2–0.5 ml) of H2O prior to further analyses.

Analytical Chromatography and Electrophoresis. Paper chromatography, paper electrophoresis, and Sephadex column chromatography were performed as described (4).

RESULTS

[3H]Mannose-labeled cell surface material from growing cells was mixed with [14C]mannose-labeled cell surface material from nongrowing cells, digested with Pronase, and chromatographed on Sephadex G-50. Glycopeptides from the growing cell surfaces had a slightly higher molecular weight distribution than those from the nongrowing cell surfaces (Fig. 1A). Glycopeptides (fractions 90–150) were pooled, lyophilized, and subjected to high voltage paper electrophoresis at pH 6.5, separating them into neutral species (to the right of the origin, Fig. 1B) and acidic species (to the left of the origin, Fig. 1B). There was a slightly higher ratio of neutral to acidic glycopeptides in growing compared to nongrowing cells reproducibly observed at this stage of purification. That neutral and acidic glycopeptides were different in molecular weight distribution was evident upon cochromatography of [14C]mannose-labeled neutral species and [3H]mannose-labeled acidic species on Sephadex G-50 (Fig. 1C). It is shown that the neutral species (average molecular weight 1500) were smaller than the acidic species (average molecular weight 3000) in this case (Fig. 1C) from the growing cell surfaces. The same is true for glycopeptides from nongrowing cell surfaces. Neuraminidase treatment did not convert the larger acidic species to the same size as the neutral species (data not shown), and thus the size difference does not seem to be solely dependent on those neuraminic acids susceptible to Vibrio cholerae neuraminidase.

When [3H]mannose-labeled acidic glycopeptides from growing cell surfaces were compared on Sephadex G-50 with [14C]mannose-labeled acidic glycopeptides from nongrowing cell surfaces, we observed that those from the growing cells had a larger size distribution (Fig. 2A). The difference in size distribution could be reduced, but not eliminated, by treatment of the mixed double-labeled glycopeptides with neuraminidase (Fig. 2B), where it can be seen, as in Fig. 2A, that the ratio of [3H]/[14C] on the leading edge of the peak is higher than on the trailing edge. There is also some indication that the peak distribution follows this description. Thus, in the acidic glycopeptides there is a growth-dependent alteration in the size distribution of cell surface glycopeptides which does not appear to be due solely to neuraminic acid susceptible to Vibrio cholerae neuraminidase.

When the neutral glycopeptides from growing and nongrowing cell surfaces were similarly compared, the size distributions were identical (Fig. 2C), and there appeared no growth-dependent alterations in size in these species.

Distinction of cell surface glycopeptides with endo-β-N-acetylgalcosaminidase D

Neutral and acidic glycopeptides were analyzed for their susceptibility to endo-β-N-acetylgalcosaminidase D. In the reaction mixture neuraminidase, β-galactosidase, and β-N-acetylgalcosaminidase were always included in order to facilitate the action of the endoglycosidase (4, 6). We found that neutral glycopeptides from nongrowing cells were substantially more digested than those from growing cells (Fig.

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3A). The products (fraction 50 to 60) were concluded to be oligosaccharides, since they were neutral when examined by paper electrophoresis at pH 1.9 and pH 6.5, and behaved as typical oligosaccharides upon paper chromatography. No significant difference in susceptibility to the endoglycosidase when the acidic glycopeptides from growing and nongrowing cells were examined (Fig. 3B). However, it is worthy of note that the acidic glycopeptides were more susceptible to the endoglycosidase than the corresponding neutral glycopeptides (Fig. 3A and B).

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4 We note that a low-molecular-weight product was released from nongrowing but not from growing acidic glycopeptides (fractions 114–118, Fig. 3B) similarly present in even greater amounts in W138 cells.
Fig. 2. Size of acidic and neutral mannosylglycopeptides from growing and density-inhibited cells. (A) Purified surface acidic glycopeptides from growing and nongrowing cells (see Fig. 1 and text for details) labeled with $^3$H and $^{14}$C, respectively, were compared on Sephadex G-50. (B) Similarly purified surface acidic glycopeptides (mixed) from growing and nongrowing cells were digested with neuraminidase from Vibrio cholerae. The mixed glycopeptides were incubated with 25 µl of neuraminidase in 0.2 ml of 0.05 M citrate buffer (pH 6.0) containing 0.15 M NaCl at 37°C for 15 hr with a small amount of toluene. For further details see ref. 4. The neuraminidase-digested mannosylglycopeptides were then compared on Sephadex G-50. (C) Purified surface neutral glycopeptides from growing and nongrowing cells were also analyzed by Sephadex G-50 column chromatography. O, $^3$H-labeled mannosylglycopeptides from growing cells; $\bullet$, $^{14}$C-labeled mannosylglycopeptides from nongrowing cells. Fractions (0.40–0.45 ml) were collected. Blue dextran (left) and fucose (right) eluted in the tubes indicated by the arrows.

Distinction of cell surface glycopeptides with α-mannosidase

When $^3$H/mannose-labeled cell surface glycopeptides (not fractionated into neutral and acidic species) from nongrowing cells and $^{14}$C/mannose-labeled cell surface glycopeptides from growing cells were mixed and digested with α-mannosidase, we found that more mannose was released from those derived from growing cells (Table 1, column 1). An isotope effect was excluded since reversing the labels did not change the result (data not shown). Since α-mannosidase from jack bean meal is a strict exoglycosidase, acting on mannose residues exposed at the nonreducing terminus (9), we conclude that there are more terminal mannosyl residues or clusters of this sugar terminating in mannose in glycopeptides of growing cells compared to those of nongrowing cells.

The differential susceptibility toward α-mannosidase persisted even after separation of the glycopeptides into neutral and acidic fractions (Table 2). The data on the neutral glycopeptides are of particular interest, since the increased susceptibility of the glycopeptides from growing cells to α-mannosidase was an apparent mirror image of their decreased susceptibility to the endoglycosidase (Fig. 3A). Furthermore, neutral glycopeptides were generally more susceptible to α-mannosidase than the corresponding acidic glycopeptides (Table 2). This again contrasted with the susceptibility toward the endoglycosidase, which is more active on acidic glycopeptides (Fig. 3B). This complementary relationship between the susceptibility to the endoglycosidase and α-mannosidase prompted us to perform the following experiment.

When the glycopeptide preparations were first incubated with α-mannosidase, heated, and then incubated with the endoglycosidase in the presence of the three exoglycosidases, the degree of the oligosaccharide release was generally unchanged as compared to the result without prior α-mannosidase digestion (Table 1, compare column 1 with 2 and column 3 with 4). Thus, we concluded that the α-mannosidase-resistant structure and the endoglycosidase-resistant structure were probably distinct species. Moreover, the sum of the released mannose and the released oligosaccharides comprise 90% of the total mannose label (Table 1, columns 2 and 4), indicating that the two types of structure comprise the majority of the mannose-labeled glycopeptide species of
Glycopeptides were digested with \( \alpha \)-mannosidase or with endo-\( \beta \)-N-acetylglucosaminidase \( \text{D} \) and the reaction was carried out as described (4). Upon paper chromatography, all of the radioactivity was found in oligosaccharides or at \( R_{\text{gelatine}} \) of 0.5 or 0.4. Glycopeptides were digested with \( \alpha \)-mannosidase or with endoglycosidase or with both. The digested material was applied to Whatman 3 MM paper and developed by butanol/acetic acid/1 M \( \text{NH}_4\text{OH} \) (2:3:1). The paper was cut into 1-cm strips and radioactivity was determined. Results are expressed as per cent of radioactivity in the products per total radioactivity recovered on the paper. For \( \alpha \)-mannosidase digestion, glycopeptides (1,000–40,000 cpm) were digested with 0.7 unit of \( \alpha \)-mannosidase in 0.2 ml of 0.1 M acetate buffer, pH 4.0, containing 0.01 M \( \text{ZnSO}_4 \). The reaction was continued for 15 hr at 37\( ^\circ \) under a toluen layer. For endoglycosidase digestion, glycopeptides (1,000–40,000 cpm) were incubated as described (4). Upon paper chromatography, all of the radioactivity was found in oligosaccharides or at \( R_{\text{gelatine}} \) of 0.5 or 0.4, or at the origin. The data presented in columns 2 and 4 were obtained as follows: \( \alpha \)-mannosidase digestion was carried out in 0.05 M citrate-phosphate buffer, pH 4.0, containing 0.01 M \( \text{ZnSO}_4 \). After the reaction, the digest was heated at 100\( ^\circ \) for 1 min, and then the pH of the reaction mixture was brought to 6.0 by the addition of 1 M \( \text{NaOH} \). Then endoglycosidase, \( \beta \)-galactosidase and \( \beta \)-N-acetylglucosaminidase, and neuraminidase were added and the reaction was carried out as above.

The digestion with \( \alpha \)-mannosidase was carried out as described in Table 1.

### DISCUSSION

We have described growth-dependent pattern changes of mannose-labeled glycopeptides from the surfaces of human diploid cells maintained strictly in the growing or nongrowing state (4). The neutral glycopeptides from growing cells...
were more resistant to endo-β-N-acetylglucosaminidase D and were more susceptible to α-mannosidase than those from nongrowing cells. The key to understanding the structural basis of this difference is the strict specificity of the endoglycosidase with respect to the oligomannosyl cores in the substrates (6, 12). In nonmembrane glycopeptides, glycopeptides with simple oligomannosyl cores have side chains with the sequence of (sialic acids) → galactose → N-acetylglucosamine which cover the oligomannosyl cores. On the other hand, glycopeptides with complex oligomannosyl cores do not have side chains and thus have exposed oligomannosyl cores (5). The endoglycosidase generally hydrolyzes glycopeptides with simple oligomannosyl cores, when the cores are exposed by pretreatment with exoglycosidases (6, 12). α-Mannosidase acts on exposed oligomannosyl cores; thus, in nonmembrane glycopeptides it acts on glycopeptides with the complex cores but not on those with the simple cores. In other words, susceptibility to the endoglycosidase and that to α-mannosidase are in complementary relationship in nonmembrane glycopeptides. We found exactly the same relationship in the mannosylglycopeptides from the surface of KL-2 cells. Thus, we concluded that the above-mentioned growth-dependent alteration in the neutral glycopeptides is due to alterations in oligomannosyl cores and suggest that compared to nongrowing cells, the surface material from growing cells is enriched in glycopeptides with complex and exposed oligomannosyl cores such as the majority of those found in ovalbumin glycopeptides (6, 13) and Unit A glycopeptides of thyroglobulin (5). It may be meaningful to point out that resistance to the endoglycosidase can occur not only by the complexity of the cores but also by their incomplete exposure (12). However, we excluded the latter possibility since the endoglycosidase-resistant structure was more susceptible to α-mannosidase, indicating that the resistant structure generally had exposed oligomannosyl cores even without pretreatment with exoglycosidases.

Another growth-dependent alteration was found in the acidic glycopeptide fraction resistant to the endoglycosidase. This difference (Fig. 4D) was probably based on the difference in the peptide moiety near the protein-carbohydrate linkage region. By analyzing fucose-labeled glycopeptides, we had previously demonstrated a growth-dependent alteration near the protein carbohydrate linkage region in glycopeptides susceptible to the endoglycosidase (4). In both cases, the material from the surface of nongrowing cells showed more complex profiles than those from growing cells. Therefore, the two pattern changes might indicate that new species of glycoprotein appeared or new carbohydrate chains were added in preexisting (glyco)proteins, during the process of density-dependent inhibition of growth. The emergence of a new galactoprotein on the surface of nongrowing cells (10) may well be related to our observations.

Growth-dependent size differences of fucose-labeled glycopeptides have already been well established (1–3) and are ascribed to the difference of the number of sialic acids (3, 11). The size change of mannosylated acidic glycopeptides observed in the present experiment (Fig. 2A) may be analogous to the above alteration. However, the failure of the complete removal of the difference by neuraminidase (Fig. 2B) is of considerable interest, and may again indicate the complex nature of this size difference. The possible relationship of these changes to growth regulation in cultures is unknown.

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