ANALYSIS OF OLIGOMANNOSYL CORES OF CELLULAR GLYCOPEPTIDES BY DIGESTION WITH ENDO-β-N-ACETYLGLUCOSAMINIDASES

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Received July 23,1975

SUMMARY Most of mannose-labeled glycopeptides from SV-40 transformed fibroblasts were hydrolyzed either by endo-β-N-acetylglucosaminidase D in the presence of β-galactosidase, β-N-acetylglucosaminidase and neuraminidase or by endo-β-N-acetylglucosaminidase H. The products were oligosaccharides with the probable structure of $Man_nGlcNAc$ (n=3,5,6,....). The D enzyme preferentially released smaller oligosaccharides, while the H enzyme released larger oligosaccharides. The results indicate the structural homology between oligomannosyl cores of the cellular glycopeptides and those of non-membrane glycopeptides.

Asparagine-linked carbohydrate chains of non-membrane glycoproteins usually have clusters of mannosyl residues (1), which will be referred as oligomannosyl cores. The number of mannose residues is variable according to glycoprotein species, namely 3 in the case of IgG, 5 to 6 in the case of ovalbumin and 5 to 11 in the case of Unit A chains of thyroglobulin (1). Since analysis of oligomannosyl cores required considerable amounts of material, the cores were studied only in limited cases in glycoproteins from cell-surfaces or from intracellular membranes (1-5).

Two types of endo-β-N-acetylglucosaminidases, *i. e.* endo-β-N-acetylglucosaminidase D (6) and endo-β-N-acetylglucosaminidase H (7, 8), have been recently introduced as new tools for the analysis of asparagine-linked carbohydrates of glycoproteins. These enzymes cleave di-N-acetylchitobiose structure in the carbohydrate chains and release oligosaccharides containing oligo-

mannosyl cores. In this communication, we will demonstrate that the combined use of the two endoglycosidases greatly facilitates the analysis of oligomannosyl cores in radioactive glycopeptides from cultured cells. Results on glycopeptides from SV-40 transformed fibroblasts will be shown as a typical example.

MATERIALS AND METHODS

Endo-β-N-acetylglucosaminidase D (6), β-galactosidase, β-N-acetylglucosaminidase and neuraminidase (9) from *Diplococcus pneumoniae*, and endo-β-N-acetylglucosaminidase H from *Streptomyces griseus* (7,8) were prepared as described previously (6,8). α-Mannosidase from jack bean meal was prepared according to Li and Li (10). Paper chromatography was carried out in ethyl-acetate-pyridine-water (12:5:4). Conditions of paper electrophoresis and liquid scintillation counting have been described (6).

SV-40 transformed 3Y1B cells (rat fibroblasts) were kindly donated by Prof. G. Kimura, Tottori University (11). The cells were grown at 37° in a stoppered TD-40 flask (surface area, 40 cm^2) containing 6 ml of Eagle's minimum essential medium with 10% fetal calf serum. Cells were plated at a density of 1×10^4 cells/cm^2, and medium was exchanged daily. Three days after plating, when the cell density was 6×10^4 cells/cm^2, 50 μCi of 2-[^{3}H]mannose (1.0 Ci/mmole, Amersham Searle Co.) or 100 μCi of U-[^{14}C]glucose (55 mCi/mmole, New England Nuclear) was added. After 24 hours, culture was washed with Earle's solution and treated with 3 ml of 0.1% trypsin (Difco) in Earle's solution lacking $CaCl_2$ and $MgSO_4$ and containing 0.016% EDTA for 5 min at room temperature. No significant cell lysis was observed at this stage by trypan-blue exclution test. Glycopeptides from trypsin released material and those from the residual cell material were prepared by pronase digestion followed by Sephadex G-50 column chromatography (12) and were designed as cell-surface glycopeptides and cell-remainder glycopeptides, respectively. The ratio of cell-surface glycopeptides to cell-remainder glycopeptides was about 1:10. When [^{3}H]mannose-labeled glycopeptides were hydrolyzed with 1 N HCl at 100° for 4 hours, 90% of label was recovered as mannose upon paper chromatography. Hydrolysis with 0.1 N HCl at 100° for 45 min detected conversion of 5% of radioactivity into fucose.

RESULTS AND DISCUSSION

[^{3}H]Mannose-labeled glycopeptides from the cell-surface or the cell-remainder of SV-40 transformed rat fibroblasts (11) were digested with endo-β-N-acetylglucosaminidase D in the presence of β-N-acetylglucosaminidase, β-galactosidase and neuraminidase. When the reaction mixture was analyzed by paper

chromatography for 3 days, the release of heterogeneous oligosaccharides was revealed (Fig. 1-A, B). Under these conditions, intact glycopeptides stayed at the origin. The smallest oligo-

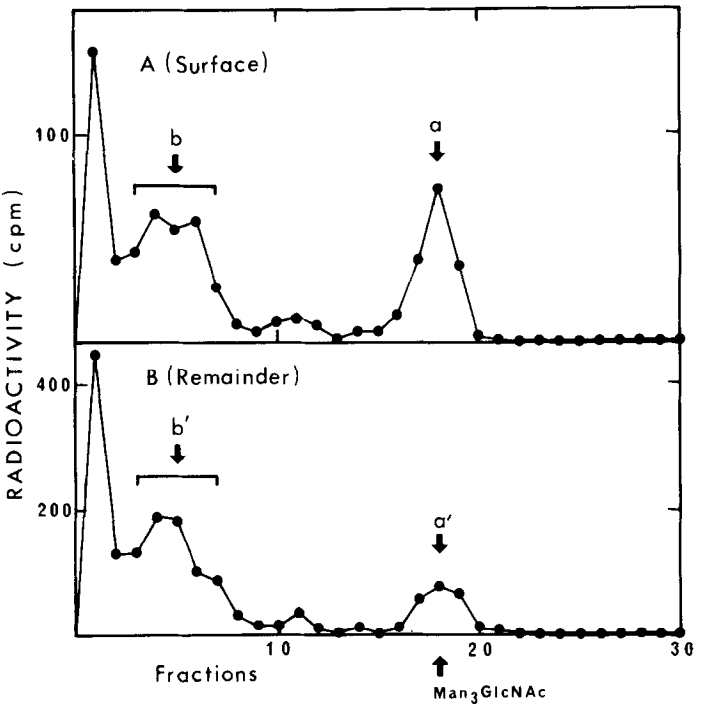

Fig. 1. Paper chromatography of the products of endo-β-N-acetylglucosaminidase D digestion.

The reaction mixture was applied to Whatman No. 1 paper and was chromatographed for 3 days. Upon shorter development, no smaller oligosaccharides nor monosaccharides could be detected. The chromatogram was cut into 1 cm pieces and was analyzed by liquid scintillation counting. Fraction 1 represents -1 to 1 cm from the origin. The standard oligosaccharide, $Man_3GlcNAc$ was prepared from bovine IgG glycopeptides (6,8) and was revealed by staining with alkaline silver nitrate.

The endoglycosidase digestion was carried out at 37° for 15 hours in 0.1 ml of 0.05 M citrate-phosphate buffer, pH 6.0 containing 5.2 miliunits of endo-β-N-acetylglucosaminidase D, 7.1 miliunits of β-galactosidase, 23 miliunits of β-N-acetylglucosaminidase and 1.8 miliunits of neuraminidase with a small amount of toluene.

A: The digestion products of mannose-labeled cell-surface glycopeptides

B: The digestion products of mannose-labeled cell-remainder glycopeptides

saccharide marked as "a" and "a'" showed identical Rf value with the tetrasaccharide, $Man_3GlcNAc$ isolated from IgG glycopeptides. After development for 10 days, the larger oligosaccharides marked as "b" and "b'" were separated into two components with the mobilities of $Man_5GlcNAc$ and $Man_6GlcNAc$ (Fig. 2-A, B). Most of the cell-surface glycopeptides were converted to oligosaccharides by the endoglycosidase digestion (Fig. 1-A, 2-A), while considerable amounts of cell-remainder glycopeptides treated with the endoglycosidase remained at the origin (Fig. 2-B). The apparently resistant material was hydrolyzed by endo-β-N-acetylglucosaminidase H, releasing two large oligosaccharides (Fig. 2-C). Although no standard oligosaccharides were available, the large oligosaccharides could be inferred to be an octasaccharide and a nonasaccharide. As above, we established that most of the cellular glycopeptides labeled with mannose were hydrolyzed either by endo-β-N-acetylglucosaminidase D or by endo-β-N-acetylglucosaminidase H.

When the intact cell-remainder glycopeptides were digested with endo-β-N-acetylglucosaminidase H, we found the release of four distinct oligosaccharides marked as "c", "d", "e" and "f" (Fig. 3-A). The larger oligosaccharides corresponded to those found in Fig. 2-C, and the smaller ones corresponded to those in Fig. 2-B. In contrast to cell-remainder glycopeptides, only small amounts of oligosaccharides were released from cell-surface glycopeptides by endo-β-N-acetylglucosaminidase H (Fig. 3-B).

The mannose-labeled oligosaccharides (fractions a, a', b, b', c, d and e in Fig. 1 and 3) were prepared in large scale experiments and were characterized. They were neutral oligosaccharides, since they did not migrate upon paper electrophoresis at pH 5.4 and 1.9. When the oligosaccharides were digested with

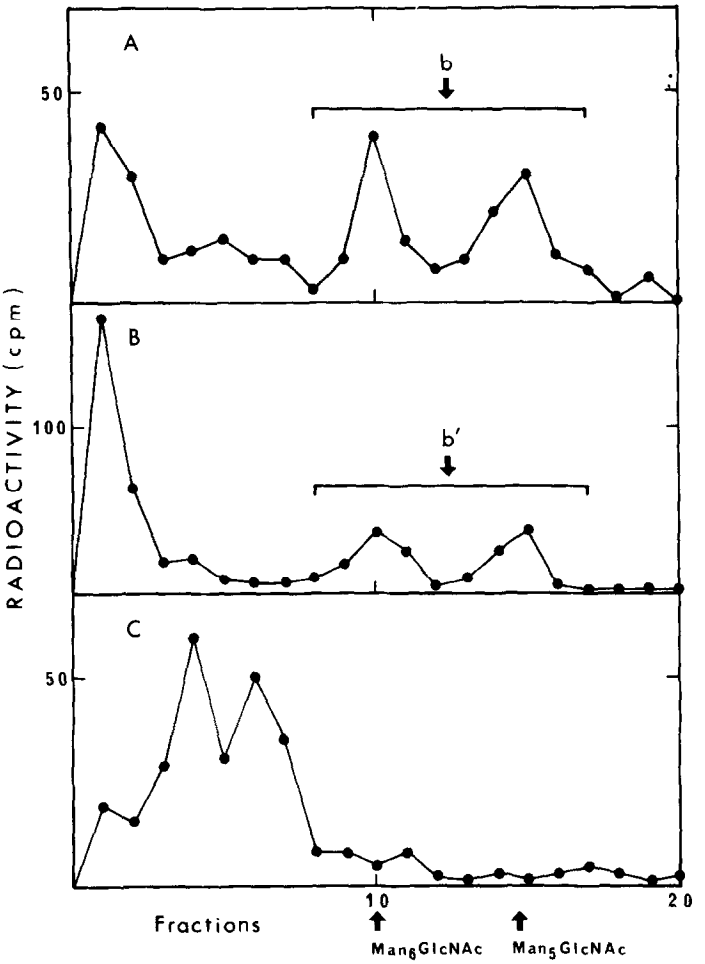

Fig. 2. Paper chromatography of the products of endo-β-N-acetylglucosaminidase D and H digestion.

The chromatogram was developed for 10 days, cut into 1.5 cm piecies and was counted. Fraction 1 represents -1.5 to 1.5 cm from the origin. The intact glycopeptides stayed at fraction 1 and 2.

The standard oligosaccharides, $Man_5GlcNAc$ and $Man_6GlcNAc$ were prepared from ovalbumin glycopeptides by digestion with the endo-β-N-acetylglucosaminidases (6,8) and were stained by alkaline silver nitrate.

Digestion with endo-β-N-acetylglucosaminidase H was carried out in 0.1 ml of 0.05 M citrate-phosphate buffer, pH 5.0 containing 9.5 miliunits of the enzyme at 37° for 15 hours with a small amount of toluene. Digestion with endo-β-N-acetylglucosaminidase D was performed as described in Fig. 1.

A: Mannose-labeled cell-surface glycopeptides were digested with endo-β-N-acetylglucosaminidase D.

B: Mannose-labeled cell-remainder glycopeptides were digested with endo-β-N-acetylglucosaminidase D.

C: Mannose-labeled cell-remainder glycopeptides resistant to endo-β-N-acetylglucosaminidase D (Fraction 1 to 2 in Fig. 2-B) were digested with endo-β-N-acetylglucosaminidase H.

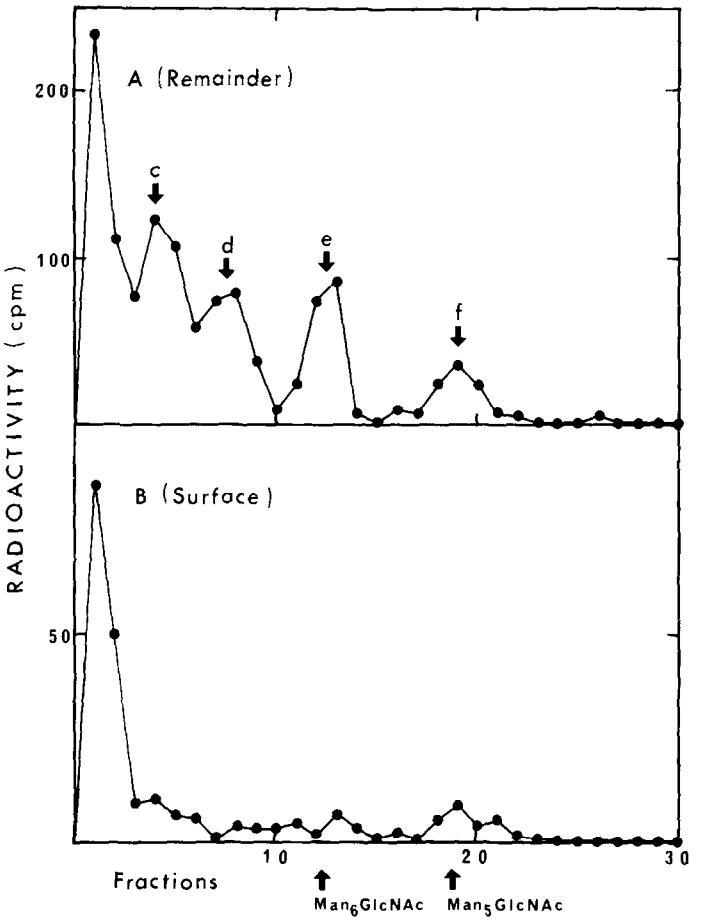

Fig. 3. Paper chromatography of the products of endo-β-N-acetylglucosaminidase H digestion.

The chromatogram was developed for 10 days, cut into 1 cm pieces and was counted. Fraction 1 represents -1 to 1 cm from the origin. The intact glycopeptides stayed at fraction 1 and 2. No smaller oligosaccharides nor monosaccharides were revealed upon shorter development. Digestion with endo-β-N-acetylglucosaminidase H was performed as described in Fig. 2.

A: The digestion products of mannose-labeled cell-remainder glycopeptides

B: The digestion products of mannose-labeled cell-surface glycopeptides.

9.0 units of α-mannosidase in 0.1 ml of 0.05 M citrate-phosphate buffer, pH 4.0 at 37° for 15 hours, and were analyzed by paper chromatography, more than 60% of the mannose label from any of

the oligosaccharides was located in the mannose region. The rest of the label was mainly located in a spot with an R_{Man} value of 0.75, which was identical with that of Manβ1→4GlcNAc isolated from IgG glycopeptides (8). When oligosaccharides prepared from [^{14}C]glucose-labeled glycopeptides were hydrolyzed with 1 N HCl at 100° for 4 hours and were analyzed by paper chromatography, more than 70% of the radioactivity co-migrated with authentic mannose, and the rest of the radioactivity was located in the glucosamine region and at the origin. Therefore, the general structure of the oligosaccharides released by the two endoglycosidases was proposed to be $Man_nGlcNAc$. From the chromatographic mobilities, the number of n was suggested to be 3, 5 and 6 for oligosaccharides released by endo-β-N-acetylglucosaminidase D. The n value could be 5, 6, 7 and 8 for those released by endo-β-N-acetylglucosaminidase H.

The above results indicated that most of mannosyl residues in cellular glycopeptides from the SV-40 transformed cells were present as oligomannosyl cores with various sizes as seen in non-membrane glycopeptides. Applicating the method described herein, we could draw the same conclusion also in KL-2 cells, human diploid fibroblasts (13). Furthermore, in KL-2 cells, growth-dependent population changes of oligomannosyl cores have been demonstrated by using the endoglycosidases (14, 13). Thus, analysis of oligomannosyl cores by the present method in various cell lines with different properties is expected to offer information relevant to biological implications of the cores in membrane glycoproteins, which are synthesized through lipid intermediates (5).

ACKNOWLEDGEMENTS We wish to express our sincere gratitude to Prof. A. Kobata for his generous support and helpful advice. We are also indebted to Prof. G. Kimura, Tottori University for SV-

3Y1B cells. An expert secretarial assistance of Miss M. Inohara is also appreciated. This work was supported by Jane Coffin Childs Memorial Fund for Medical Research, Damon Runyon Memorial Fund for Cancer Research and Scientific Research Funds of the Ministry of Education, Japan.

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