A Novel Endo-β-galactosidase from Clostridium perfringens That Liberates the Disaccharide GlcNAcα1→4Gal from Glycans Specifically Expressed in the Gastric Gland Mucous Cell-type Mucin*

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We found that commercially available sialidases prepared from Clostridium perfringens ATCC10543 were contaminated with an endoglycosidase capable of releasing the disaccharide GlcNAcα1→4Gal from glycans expressed in the gastric gland mucous cell-type mucin. We have isolated this enzyme in electrophoretically homogeneous form from the culture supernatant of this organism by ammonium sulfate precipitation followed by affinity chromatography using a Sephacryl S-200 HR column. The enzyme was specifically retained by and eluted from the column with methyl-α-Glc. By NMR spectroscopy, the structure of the disaccharide released from porcine gastric mucin by this enzyme was established to be GlcNAcα1→4Gal. The specificity of this enzyme as an endo-β-galactosidase was established by analyzing the liberation of GlcNAcα1→4Gal from GlcNAcα1→4Galβ1→6GlcnAcβ1→4Galβ1→3GalNAc-ol by mass spectrometry. Because this novel endo-β-galactosidase specifically releases the GlcNAcα1→4Gal moiety from porcine gastric mucin, we propose to call this enzyme a GlcNAcα1→4Gal-releasing endo-β-galactosidase (Endo-β-GalGna). Endo-β-GalGna was found to remove the GlcNAcα1→4Gal epitope expressed in gastric adenocarcinoma AGS cells transfected with α1,4-N-acetylgalactosaminyltransferase cDNA. Endo-β-GalGna should become useful for studying the structure and function of glycoconjugates containing the terminal GlcNAcα1→4Gal epitope.

Clostridium perfringens, a ubiquitous anaerobic bacterium commonly found in the gastrointestinal tract of higher animals and in soil, has been known to cause a wide variety of diseases in man and animals (1–3). The pathogenesis caused by C. perfringens has been known to cause a wide variety of diseases commonly found in the gastrointestinal tract of higher animals and in soil, has been known to cause a wide variety of diseases in man and animals (1–3). The pathogenesis caused by C. perfringens perfringens infection has been attributed to the toxins includ-

EXPERIMENTAL PROCEDURES

Materials—The hexasaccharide-alditol, GlcNAcα1→4Galβ1→4GlcNAcβ1→6GlcNAcβ1→3GalNAc-ol was isolated from PGM according to the procedure described by Ishihara et al. (7). The structure of this hexasaccharide-alditol was confirmed by 1H and 13C NMR spectroscopy (data not shown). Keratan sulfate was isolated from the nasal cartilage of sei whale, Balaenoptera borealis (9). β-N-Acetylbiosaminidase (β-Hex) was isolated from jack bean meal (10). The following specific chemicals and reagents were purchased from the commercial sources indicated. PGM (type II), heparin, heparan sulfate, methyl-α-Glc, and peroxidase-conjugated anti-mouse IgG were from Sigma. Anti-mucin mAb (HIK1083) that recognizes the terminal α-linked GlcNAc was from Kanto Chemical (Tokyo, Japan). Galβ1→4GlcNAc, Galβ1→4GlcNAcβ1→3Glcβ1→4Glc, Azocoll, and anti-Tn antigen (GlcNAcα1→3Galβ1→4Glc) mAb were from

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† The abbreviations used are: PGM, porcine gastric mucin; Endo-β-GalGna, GlcNAcα1→4Gal-releasing endo-β-galactosidase; β-Hex, β-N-acetylbiosaminidase; mAb, monoclonal antibody; Galβ1→4GlcNAc, N-acetyllactosamine; HPLC, high performance liquid chromatography; COSY, homonuclear shift correlated spectroscopy; ESI-MS, electrospray ionization-mass spectrometry; αGalGN, α1,4-N-acetylgalactosami-

nysynthesizing enzyme; HSQC, 1H-detected heteronuclear single-quantum coherence; ROESY, rotating frame Overhauser effect spectroscopy; HMBC, 1H-detected heteronuclear multiple-bond correlation; NOE, nuclear Overhauser effect.

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Calbiochem. Blood group A- and B-pentasaccharides, GalNAcα1→3(Fucα1→2)Galβ1→4(Fucα1→3)Glc, and blood group B-pentasaccharide, Galα1→3(Fucα1→2)Galβ1→4(Fucα1→3)Glc, were from Glycotech (Rockville, MD). Fluorescein isothiocyanate-conjugated anti-mouse IgM was from Immunotech (Marseille, France). All other general chemicals were from commercial sources and of the highest grade.

Enzyme Assay—PGM that had been dialyzed exhaustively against distilled water was used as a substrate for assaying the Endo-β-GalGalNAc activity. The 10-μl reaction mixture containing 80 μg of PGM and an appropriate amount of enzyme in 20 mM sodium acetate buffer, pH 6.0 (Buffer A), was incubated at 37 °C for a predetermined time. The reaction was stopped by adding 2 μl of glacial acetic acid. An aliquot of the reaction mixture was applied to a silica gel TLC plate (Merck, Germany) and developed twice with 1-butanol:acetic acid:water (2:1:1, v/v/v), sprayed with the diphenylamine-aniline-phosphoric acid reagent, and heated at 110 °C for 20 min to visualize glycoconjugates (11). The quantitative analysis of each glycoconjugate band on a TLC plate was carried out using a Scan Jet 2C/ADF scanner (Hewlett-Packard) and the NIH Image 1.61 program. One unit of enzyme activity was defined as the amount that releases 1 μmol of GlcNAcα1→4Gal from PGM/min at 37 °C. N-Acetyllactosamine (Galβ1→4GlcNAc) was used as a standard to calibrate the intensity of the band. The standard curve was linear up to 0.5 μg of N-acetyllactosamine/band.

Purification of Endo-β-GalGalNAc—All operations for the enzyme isolation were carried out at 0–5 °C. C. perfringens ATCC10543 obtained from American Type Culture Collection was cultured under anaerobic conditions at 37 °C for 24 h in 3.5% Todd-Hewitt broth (Difco) supplemented with 0.18% K2HPO4, 0.25% NaCl, 0.15% glucose, and 0.005% Cys (12). After centrifugation at 12,000 × g for 30 min, the culture supernatant (2.3 liters) was brought to 80% saturation with solid ammonium sulfate and left standing overnight at 4 °C. The precipitate was collected by centrifugation at 12,000 × g for 30 min, dissolved in 15 ml of Buffer A, and dialyzed against the same buffer. This crude enzyme preparation was applied on a Sephacryl S-200 HR column (2.5 × 47 cm, Amersham Pharmacia Biotech) previously equilibrated with Buffer A. The column was washed with Buffer A following by washing with Buffer A containing 0.5 M NaCl to remove the unasorbed proteins. The enzyme retained by the column was eluted with 50 mM methionyl-α-Glc in Buffer A containing 0.5 M NaCl. Under this condition, Endo-β-GalGalNAc was the most active peak of the column. The active fractions were concentrated by Centricon YM-10 (Millipore) and dialyzed against Buffer A to remove methionyl-α-Glc and NaCl. Table I summarizes the purification of Endo-β-GalGalNAc from 2.3 liters of C. perfringens culture supernatant.

Isolation of the Product Released from PGM by Endo-β-GalGalNAc—PGM (10 mg) predialyzed against distilled water was incubated with 50 milliliters of the enzyme in 1 ml of Buffer A at 37 °C for 24 h. The reaction mixture was filtered through a Centricon YM-10 (Millipore), and the filtrate was applied onto a Sephadex G-25 SF column (1.0 × 110 cm, Amersham Pharmacia Biotech). The column was eluted with water at 4 ml/h, and 1-ml fractions were collected. The disaccharide in the effluent was monitored by TLC as described under “Enzyme Assay.” The fractions containing the disaccharide were collected and evaporated to dryness. This preparation was further purified by HPLC using a reversed phase column (Deltapak C18-300A, 3.9 × 300 mm, Waters). Elution was carried out with distilled water at a flow rate of 1.0 ml/min using a Waters 600E HPLC system. The UV absorption of the effluent was monitored at 214 nm. The peak containing the disaccharide at the retention time of 2.9 min was collected and evaporated to dryness. By this procedure, 0.4 mg of the pure disaccharide was obtained from 10 mg of PGM.

NMR Analyses—For NMR analyses, the disaccharide (0.4 mg) isolated by HPLC was exchanged repeatedly with D2O, with intermediate lyophilization and then dissolved in 250 μl of D2O. The NMR spectra were recorded at 25 ± 0.1 °C on a General Electric Omega PSG 500 MHz spectrometer. A 3-mm inverse probe (Nolarc, Martinez, CA) was used for all measurements. The 1H and 13C signal assignments rely on the initial delineation of the individual proton spin systems within a molecule using the double quantum-filtered COSY (13, 14), total correlation spectroscopy (15, 16), nuclear Overhauser enhancement in rotating frame (17), indirectly detected heteronuclear single-bond 1H-13C correlation (18), and 1H-detected heteronuclear multiple bond connectivity (19, 20).

ESI-MS Analysis—The dried samples were dissolved in methanol: water (1:1, v/v) to give a final concentration of 100 μg/ml. ESI-MS was performed in the positive ion mode, employing a Micromass Quattro II mass spectrometer (Cheshire, U. K.). A Harvard syringe pump (Holliston, MA) was used to deliver sample solutions directly into the ESI source at 5 μl/min. Nitrogen was used as nebulizing gas and drying gas at flow rates of 15 and 350 liters/min, respectively. The source temperature was set at 80 °C. The ESI capillary voltage was held at ~2.8 kV, and the cone voltage was set at 20 V. During all MS analyses, the analyzer pressure was kept at ~1 × 10−5 mBar. Collected data were processed using MassLynx 3.0 software. All spectra shown in this paper represent signal-averaged spectra of at least 25 scans without further processing.

Dot blot Immunostain Analysis—PGM (250 μg) was incubated with or without 100 milliliters of Endo-β-GalGalNAc in 500 μl of Buffer A at 37 °C for 24 h. Half of each reaction mixture was incubated further with 100 milliliters (2 μl) of β-Hex at 37 °C for 6 h. Two μl from each mixture containing 1.0 μg PGM was applied directly onto a nitrocellulose membrane (Bio-Rad) and then the membrane was incubated with anti-Tn antigen (GalNAcα1→3Ser/Thr) mAb (1:50). Peroxidase-conjugated anti-mouse IgG (1:1,000) was used as the secondary antibody, and 4-chloro-naphthol was used as the chromogenic substrate for peroxidase.

Immunohistochemistry of AGS-α4GnT Cells—Human gastric adenocarcinoma AGS-α4GnT cells stably expressing GlcNAcα1→4Galβ1→R in O-glycans on their cell surface were established by cotransfection of AGS cells with pcDNAI-α4GnT and pSV2neo. pcDNAI-α4GnT expresses human α4GnT, a transferase responsible for the biosynthesis of GlcNAcα1→4Galβ1→R, and pSV2neo encodes a G418 resistance gene (8). These cells were grown on Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) and fixed with 20% buffered formalin, pH 7.4, for 15 min. After washing with phosphate-buffered saline, the AGS-α4GnT cells were incubated with 300 milliunits of Endo-β-GalGalNAc in 500 μl of phosphate-buffered saline at 37 °C for 24 h and then subjected to the immunohistochemical analysis using HIK1083 mAb specific for GlcNAcα1→4Galβ1→R as described previously (8). For a control experiment, digestion with Endo-β-GalGalNAc was omitted from the procedure. Fluorescein isothiocyanate-conjugated anti-mouse IgM was used as the secondary antibody, and Vectoashield (Vector Laboratories, Burlingame, CA) was used for mounting the slides. The immunopelabeling was analyzed using a confocal laser scanning microscopy LSM510 (Carl Zeiss, Jena, Germany).

Other Methods—N-terminal sequencing of Endo-β-GalGalNAc was carried out by the Core Facility of Louisiana State University, Health Sciences Center, New Orleans, LA. Exoglycosidases were assayed using 2 mM of various p-nitrophenyl glycosides in 50 mM sodium acetate buffer, pH 6.0, as described previously (21). Protease activity was assayed using Azocoll as substrate (22). The protein concentration was determined using the BCA protein assay reagent (Pierce) with bovine serum albumin as a standard. Sugar compositions of oligosaccharides were analyzed by a high performance anion exchange chromatography equipped with a pulsed amperometric detector (HPAEC-PAD system) using a CarboPac PA-1 column (Dionex, Sunnyvale, CA).

RESULTS

Facile Purification of Endo-β-GalGalNAc—As described under “Experimental Procedures,” Sephacryl S-200 HR was found to be a very effective affinity matrix for one-step purification of Endo-β-GalGalNAc from the crude enzyme prepared from the culture supernatant of C. perfringens. Endo-β-GalGalNAc was

| Step | Protein | Total activity | Specific activity | Yield | Purification |
|------|---------|----------------|-----------------|-------|-------------|
| Culture supernatant | 1,571 | 22.0 | 0.014 | 100 | 1 |
| Ammonium sulfate | 992 | 16.6 | 0.017 | 75.5 | 1.2 |
| Sephacryl S-200 HR | 0.24 | 11.5 | 47.9 | 52.3 | 3,420 |
purified 3,000-fold with an overall recovery of about 50% (Table I). As shown in Fig. 1, the purified enzyme migrated as a single protein band of about 46 kDa in SDS-polyacrylamide gel electrophoresis. A single N-terminal peptide sequence, KDFPAN-PIEKAGYKLD, was obtained for this enzyme. No exoglycosidase and protease activities were detected in the final preparation of Endo-β-Gal_{GlcNAc}.

General Properties of Endo-β-Gal_{GlcNAc}—The effect of pH on the activity of Endo-β-Gal_{GlcNAc} was examined by using the following buffers at a concentration of 20 mM: sodium acetate, pH 3.5–6.0; sodium phosphate, pH 6.0–7.5; Tris-HCl, pH 7.5–9.0, and sodium borate, pH 9.0–10.5. The optimum pH of the enzyme was found to be pH 6.0 in sodium acetate buffer (Buffer A), and the enzyme retained more than 80% of its activity between pH 5.5 and 7.0. The enzyme did not lose any activity at 4 °C for longer than 3 months or at 37 °C for 72 h in Buffer A.

Among various divalent metal ions tested at a 10 mM concentration, only Mg^{2+} inhibited 60% of the enzyme activity, whereas Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+} had no effect on the activity. Neither 10 mM EDTA nor 100 mM 2-mercaptoethanol affected the enzyme activity. The enzyme was remarkably stable in SDS. No loss of the activity was observed in 0.2% SDS, and the enzyme retained 60% of its activity in 1.0% SDS.

Liberation of GlcNAc1→4Gal from PGM by Endo-β-Gal_{GlcNAc}—The crude enzyme prepared from the culture supernatant of C. perfringens released several monosaccharides and oligosaccharides from PGM (Fig. 2A, lane 4). The purified Endo-β-Gal_{GlcNAc} on the other hand, released only one oligosaccharide from PGM (Fig. 2A, lane 5). Using a Dionex HPAEC-PAD analyzer, this oligosaccharide was found to contain GlcNAc and Gal in an equimolar ratio. The structure of this oligosaccharide was subsequently established by NMR spectroscopy.

Table II summarizes the assignments of 1H and 13C NMR chemical shifts together with important coupling constants for the oligosaccharide released from PGM. The primary structure of this oligosaccharide was identified to be GlcNAc1→4Gal based on the following observations. The Gal residue at the reducing end of this disaccharide existed as a 7:3 mixture of α- and β- anomers exhibiting two sets of proton and carbon signals for Gal (Fig. 2B and Table III). The anomeric linkage of the GlcNAc residue of the disaccharide was found to have an α-configuration by recording an HSQC spectrum of the disaccharide without decoupling. From this spectrum, the one-bond C-H coupling constant for the α-GlcNAc was determined to be 171.0 Hz, in contrast to 160.7 Hz for the β-Gal unit of this disaccharide (Table II). In the ROESY spectrum, the anomeric proton of α-GlcNAc residue shows a strong cross-peak with the Gal-H4 as well as an intra-ring effect at H2. The latter effect is characteristic of an α-anomeric linkage. The α-anomeric configuration of GlcNAc is also supported by the small vicinal coupling constant (3.1 Hz) observed for J_3,2 as shown in Table II.

In the disaccharide released from PGM, C4 of the α- (77.99 ppm) and β-Gal (77.07 ppm) residues have moved downfield by more than 7 ppm in respect to the corresponding methylgalactopyranosides. These indicate that C4 of Gal is substituted. The results of ROESY experiments (shown in Table II) suggest that the linkage between GlcNAc and Gal in the disaccharide is GlcNAc1→4Gal.

After completely assigning the carbon spectrum (Table II and Fig. 3A), we were able to make an unambiguous determination of the glycosidic linkage from the long range C-H correlation (HMBC). The inter-residue correlations observed in the HMBC spectra of the disaccharide are listed in Table II and in Fig. 3, B and C. The presence of the strong cross-peaks, Gal/H4/GlcNAc-C1 and GlcNAc-H1/Gal-C4 in the HMBC spectra, establishes the GlcNAc1→4Gal interglycosidic linkage in this disaccharide.

Substrate Specificity of Endo-β-Gal_{GlcNAc}—To characterize the specificity of Endo-β-Gal_{GlcNAc} further, we used TLC and MS to analyze the hydrolysis of GlcNAc1→4Galβ1→4GlcNAcβ1→6GlcNAcβ1→3GalNAc-ol by this enzyme. As expected, this hexasaccharide-alditol was refractory to jack bean α-Hex (Fig. 4A, lane 3). However, Endo-β-Gal_{GlcNAc} converted the hexasaccharide-alditol to an oligosaccharide and a disaccharide with TLC mobility identical to that of the authentic GlcNAc1→4Gal (Fig. 4A, lane 5). Incubation of the hexasaccharide-alditol with both Endo-β-Gal_{GlcNAc} and β-Hex resulted in the conversion of the slow moving oligosaccharide to
NMR chemical shifts are given in ppm from the HSQC spectrum obtained at 500 MHz in D2O at 25.0 °C ± 0.1 °C. The 1H chemical shifts are expressed relative to HOD (4.78 ppm at 25 °C, δ acetone 2.25 ppm). Even under strict temperature control, 1H chemical shifts are not reproducible to three decimals; however, within a given experiment at 500 MHz, signals can be distinguished accurately to this precision. The 13C chemical shifts are relative to 1,4-dioxane, using the deuterium lock of the spectrometer, which sets the chemical shift for dioxane at 66.84 ppm. Coupling constants are given in Hz.

| Position | 1H | 3JHH | 3JC | 1JCH | Interglycosidic HMBC/ROESY |
|----------|----|------|-----|------|--------------------------|
| aGlcNAcαGal | 4.837 | 3.1 | 98.38 | 171.0 | βGal-H4/aGlcNAc-Cl; aGlcNAc-H1/βGal-H4 |
| 1 | 3.863 | 54.04 | | | |
| 2 | 3.746 | 70.45 | | | |
| 3 | 3.500 | 69.63 | | | |
| 4 | 4.145 | 71.98 | | | |
| 5 | 3.723, 3.791 | 59.95 | | | |
| Ac-CH3 | 2.047 | 21.83 | | | |
| Ac-CO | 175.00 | | | | |
| aGlcNAcβGal | 4.837 | 3.1 | 98.38 | 171.0 | aGal-H4/aGlcNAc-Cl; aGlcNAc-H1/αGal-H4 |
| 1 | 3.863 | 54.04 | | | |
| 2 | 3.746 | 70.45 | | | |
| 3 | 3.500 | 69.63 | | | |
| 4 | 4.145 | 71.98 | | | |
| 5 | 3.723, 3.791 | 59.95 | | | |
| Ac-CH3 | 2.047 | 21.83 | | | |
| Ac-CO | 175.00 | | | | |
| βGal | 4.602 | 7.8 | 96.65 | 160.7 | |
| 2 | 3.476 | 71.67 | | | |
| 3 | 3.864 | 72.28 | | | |
| 4 | 3.922 | 77.07 | | | |
| 5 | 3.699 | 75.34 | | | |
| 6 | 3.711, 3.640 | 60.26 | | | |
| aGal | 5.259 | 3.7 | 92.36 | 169.3 | |
| 1 | 3.981 | 77.99 | | | |
| 2 | 3.887 | 68.62 | | | |
| 3 | 3.858 | 68.62 | | | |
| 4 | 4.088 | 71.16 | | | |
| 5 | 3.746, 3.699 | 60.26 | | | |

a aGlcNAcαGal the α-GlcNAc residue that is linked to β-Gal; aGlcNAcβGal, the α-GlcNAc residue that is linked to α-Gal.

A fast moving oligosaccharide with concomitant release of GlcNAc (Fig. 4A, lane 6). These results indicate that the enzyme released the GlcNAc1→4Gal linked to the core 2 branched GlcNAc through a β,1,4 linkage. This conclusion was supported further by ESI-MS analyses.

Fig. 4B1 shows the ESI-MS of the substrate hexasaccharide-alditol (monoisotopic molecular mass = 1,156.45 Da). The singly protonated hexasaccharide-alditol appears at m/z 1157.4, whereas the sodium adduct yields a peak at m/z 1,179.1. The doubly protonated form of the substrate, [M + 2H]2+ constitutes the base peak at m/z 579.3, and the sodium adduct ion can be assigned as [M + Na + H]2+ at m/z 590.3, [M + K + H]2+ at m/z 598.2, and [M + 2Na]2+ at m/z 601.2. Fig. 4B2 shows the ESI-MS of the products after the substrate has been digested with Endo-β-GalGlcGlcGlc. The structures and the monoisotopic molecular masses of the substrate and products of this reaction are shown in Fig. 4C. As expected from the result of TLC analysis, two products were observed. The peak at m/z 384.2 corresponds to a protonated GlcNAc1→4Gal (583.14 Da), whereas the peak at m/z 792.1 corresponds to the protonated counterpart of the digested substrate (791.31 Da). Sodium adducts of these two compounds appear at m/z 406.2 and 814.1, respectively, whereas no peaks for Endo-β-GalGlcGlcGlc are observed in this mass range. The ESI-MS spectrum of the products, generated by incubating the substrate with Endo-β-GalGlcGlcGlc and β-Hex, is shown in Fig. 4B3. Fig. 4C also shows the structures and the monoisotopic molecular masses of the products released from the substrate by Endo-β-GalGlcGlcGlc and β-Hex. Notably, two new compounds appeared at m/z 222.2 (protonated form, sodium adduct at m/z 244.2) and m/z 589.2 (protonated form, sodium adduct at m/z 611.1) with the disappearance of the product peaks (m/z 792.1 and 814.1) in Fig. 4B2. The GlcNAc1→4Gal peaks (m/z 384.2 and 406.2) remain. Because β-Hex cleaves the terminal β-linked GlcNAc, we can assign the peak at m/z 222.2 as protonated GlcNAc (221.09 Da) and the peak at m/z 589.2 as protonated GlcNAc1→4Galβ1→3GalNAc-ol (588.22 Da). The above results prove conclusively that Endo-β-GalGlcGlcGlc has released the GlcNAc1→4Gal moiety from the GlcNAc1→4Galβ1→3GalNAc-branch in the core 2 O-glycan in preference to the GlcNAc1→4Galβ1→3GalNAc-ol in the core 1 O-glycan of the hexasaccharide-alditol.

Although the GlcNAc1→4Galβ1→3GalNAc-ol branch was resistant to Endo-β-GalGlcGlcGlc, the aglycon, GalNAc-ol, was not in a pyranose form. To see whether the enzyme was capable of acting on the GlcNAc1→4Galβ1→3GalNAc-1→Ser/Thr in PGM, we carried out a dot-blot immunostain analysis of PGM treated with Endo-β-GalGlcGlcGlc using anti-Tn antigen mAB. As shown in Fig. 5, A and B, both the native and β-Hex-treated PGM were Tn antigen-negative, and the Endo-β-GalGlcGlcGlc-treated PGM was weakly positive (Fig. 5C). The PGM treated with both Endo-β-GalGlcGlcGlc and β-Hex, on the other hand, gave a strongly positive reaction with anti-Tn antigen mAB (Fig. 5D), indicating the exposure of GalNAc1→4Galβ1→3GalNAc sequence of the core 2 branched O-glycan but also in the GlcNAc1→4Galβ1→3GalNAc1→1Ser/Thr sequence of the core 1 structure.

The enzyme displayed Michaelis-Menten kinetics with a $K_m$...
of 0.8 mM and a $k_{cat}$ of 37 s$^{-1}$, using GlcNAc$_1$→4Gal$_{b1}$→4GlcNAc$_{b1}$→6(GlcNAc$_{b1}$→4Gal$_{b1}$→3)GlcNAc-ol as the substrate.

The endo-$b$-galactosyl linkages in the following substrates were refractory to Endo-$b$-Gal$_{Ga}$: keratan sulfate, Gal$_{a1}$→3Gal$_{b1}$→4GlcNAc$_{b1}$→3Gal$_{b1}$→4Glc, GalNAc$_{a1}$→3(Fuc$_{a1}$→2)Gal$_{b1}$→4(Fuc$_{a1}$→3)Glc, and Gal$_{a1}$→3(Fuc$_{a1}$→2)Gal$_{b1}$→4(Fuc$_{a1}$→3)Glc. The enzyme also did not liberate the blood group A trisaccharide, GalNAc$_{a1}$→3(Fuc$_{a1}$→2)Gal$_{b}$, from PGM. Heparin and heparan sulfate, which contain $a$-linked GlcNAc, were also refractory to this enzyme.

Removal of GlcNAc$_{a1}$→4Gal from the Cell Surface of AGS-$a$GnT Cells by Endo-$b$-Gal$_{Ga}$—To study the effect of Endo-$b$-Gal$_{Ga}$ upon AGS-$a$GnT cells stably expressing GlcNAc$_{a1}$→4Gal$_{b1}$→R residues, immunohistochemical analysis was performed using HIK1083 mAb. As shown in Fig. 6A, AGS-$a$GnT cells strongly reacted with HIK1083 mAb, indicating the presence of GlcNAc$_{a1}$→4Gal$_{b1}$→R residues on the cell surface. After treatment with Endo-$b$-Gal$_{Ga}$, the cells lost their ability to react with HIK1083 mAb (Fig. 6B). This result indicates that Endo-$b$-Gal$_{Ga}$ removed the disaccharide GlcNAc$_{a1}$→4Gal, an epitope recognized by HIK1083 mAb, on the cell surface of AGS-$a$GnT cells.

DISCUSSION

Commercially available sialidases have been widely used in vitro and in vivo to study the effect of desialylation of glycoconjugates. To ensure that changes after sialidase treatment are truly attributed to the removal of sialic acids, the sialidase used should be free from other contaminants. This work was initiated after an observation that commercially available sialidases prepared from C. perfringens ATCC10543 (12) were contaminated with an endoglycosidase capable of releasing a disaccharide from PGM. We have purified and characterized this unique endoglycosidase from the culture supernatant of C. perfringens. We found that this enzyme was retained by a Sephacryl S-200 HR column and could be released from the column using methyl-$a$-Glc. This step provided an effective one-step purification of this enzyme in electrophoretically homogeneous form. Using NMR spectroscopy (Figs. 2B and 3 and Table II) and mass spectrometry (Fig. 4B), we have shown unequivocally that this enzyme released GlcNAc$_{a1}$→4Gal from GlcNAc$_{a1}$→4Gal$_{b1}$→4GlcNAc$_{b1}$→6(GlcNAc$_{b1}$→4Gal$_{b1}$→3)GlcNAc-ol by specifically cleaving the internal Gal$_{b1}$→4GalNAc linkage in this hexasaccharide-alditol. Thus, this enzyme is an endo-$b$-galactosidase. However, the enzyme was not able to release GlcNAc$_{a1}$→4Gal from the GlcNAc$_{a1}$→4Gal$_{b1}$→3GlcNAc-ol branch in the hexasaccharide-alditol. Using anti-Tn antigen mAb, we were able to show indirectly that the enzyme also released GlcNAc$_{a1}$→4Gal from GlcNAc$_{a1}$→4Gal$_{b1}$→3GlcNAc-ol→Ser/Thr in PGM (Fig. 5). This may suggest that the pyranose structure of the aglycon, GalNAc, is important for the enzyme. Whether or not the enzyme is able to release GlcNAc$_{a1}$→4Gal from the GlcNAc$_{a1}$→4Gal$_{b1}$→3GlcNAc-ol sequence remains to be elucidated. Oligosaccharides containing this structure are currently not available.

Three different types of microbial endo-$b$-galactosidases have been reported. They are: (i) the endo-$b$-galactosidase that cleaves the endo-$b$-galactosyl linkages in poly lactosaminoglycans (23–27); (ii) the endo-$b$-galactosidase that releases blood group A and B trisaccharides from blood group A and B substances (28); and (iii) the endo-$b$-galactosidase (Endo-GalC) that releases the Gal$_{a1}$→3Gal from the xenoantigen Gal$_{a1}$→3Gal$_{b1}$→R (29). The endo-$b$-galactosidase presented in this report is distinct from the aforementioned three endo-$b$-galactosidases. Because this novel endo-$b$-galactosidase specifically releases GlcNAc$_{a1}$→4Gal from PGM, we propose to call this clostridial endoglycosidase a GlcNAc$_{a1}$→4Gal-releasing endo-$b$-galactosidase (Endo-$b$-Gal$_{Ga}$).

Although the $b$-linked GlcNAc is found commonly in glycoconjugates, the $a$-linked GlcNAc is relatively rare. It is well known that glycan chains of heparin and heparan sulfate contain $a$-linked GlcNAc. The attachment of an $a$-linked GlcNAc to the threonine residues of cell surface proteins in Trypanosoma...
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Halbeek et al. (34) also found the presence of this disaccharide epitope in rat duodenal gland mucin. Ishihara et al. (7) developed a mAb (HIK1083) that recognizes the terminal α-GlcNAc linked to the C4 of the Gal residues in the core 2 branched O-glycan, GlcNAc1→4Galβ1→4GlcNAcα→4Galβ1→4GlcNAc1→3GalNAc-ol.

Paradoxical concanavalin A staining, a sequential histochemical method involving periodate oxidation, sodium borohydride reduction, ConA binding, and horseradish peroxidase reaction, has been used to identify mucosubstances called class III mucin (35). In man, the occurrence of this mucin was found to be exclusively limited to the gastric gland mucous cells, such as mucous neck and pyloric gland cells, Brunner’s gland of the duodenum, and accessory gland of the pancreatobiliary tract. Thus, class III mucin is also termed gastric gland mucous cell-type mucin. Immunohistochemical analysis of the human alimentary tract indicates that HIK1083 mAb specifically reacts with class III mucin, suggesting that this mucin contains terminally linked GlcNAc in class III mucin. Class III mucin has also been found in gastric adenocarcinoma (37), pancreatic ductal carcinoma (37), mucinous bronchioalveolar cell carcinoma of the lung (38), and the adenoma malignum of the uterine cervix (39). Endo-β-GalGnT was found to remove the GlcNAc1→4Gal epitope expressed on the cell surface of gastric adenocarcinoma AGS-αGnT cells (Fig. 6). The physiological consequence of releasing the GlcNAc1→4Gal disaccharide from gastric mucin is not known at the present time. Because the GlcNAc1→4Gal-epitope is expressed only in the gastroduodenal mucin, the removal of this disaccharide from the mucin may facilitate the passage of C. perfringens from the stomach to the intestine, the primary site of infection by this bacterium. This hypothesis can be tested in the future by comparing the pathogenicity of the wild type C. perfringens with that of the mutant devoid of Endo-β-GalGnT activity.

The existence of such an unusual endo-β-galactosidase in C. perfringens ATCC10543 is very intriguing. This unique endo-β-galactosidase should become useful for studying the structure and biological function of glycoconjugates containing the GlcNAc1→4Gal epitope.

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Dot-blot analysis of PGM treated with Endo-β-GalGnT and β-Hex using anti-Tn antigen mAb. Panel A, PGM; panel B, PGM + β-Hex; panel C, PGM + Endo-β-GalGnT; panel D, PGM + Endo-β-GalGnT + β-Hex; panel E, β-Hex; panel F, Endo-β-GalGnT. Detailed experimental conditions are described under “Experimental Procedures.”

β-Hex

A

B

C

D

E

F

GlcNAcα1→4Galβ1→4GlcNAcβ1→4Galβ1→3GalNAc-ol

GlcNAcα1→4Gal + GlcNAc + GlcNAcα1→4Galβ1→3GalNAc-ol

[383,14] [221,09] [588,22]

FIG. 4. Hydrolysis of the hexaasaccharide-alditol (Hex-ol) by Endo-β-GalGnT and β-Hex. Panel A, TLC analysis. Lane 1, β-Hex; lane 2, Endo-β-GalGnT; lane 3, Hex-ol + β-Hex; lane 4, Hex-ol; lane 5, Hex-ol + Endo-β-GalGnT; lane 6, Hex-ol + Endo-β-GalGnT + β-Hex; lane 7, GlcNAcα1→4Gal; lane 8, GlcNAc. Panel B, ESI-MS analyses. Trace 1, Hex-ol; trace 2, Hex-ol + Endo-β-GalGnT; trace 3, Hex-ol + Endo-β-GalGnT + β-Hex. Panel C, hydrolysis reactions of Hex-ol catalyzed by Endo-β-GalGnT and β-Hex. The numbers in brackets represent the monoisotopic molecular mass for each component.

FIG. 5. Immunohistochemical analysis showing the effect of Endo-β-GalGnT on AGS-αGnT cells stably expressing GlcNAc1→4Gal residues on their cell surface. Panel A, immunohistochemical staining of AGS-αGnT cells with HIK1083 mAb followed by anti-mouse IgM. Panel B, immunohistochemical staining of AGS-αGnT cells that were pretreated with Endo-β-GalGnT, with HIK1083 mAb, followed by anti-mouse IgM. Panel C, immunohistochemical staining of AGS-αGnT cells with anti-mouse IgM alone. These images were analyzed using a laser confocal microscope (bar = 50 μm).
A Novel Endo-β-galactosidase from Clostridium perfringens That Liberates the Disaccharide GlcNAc α1→4Gal from Glycans Specifically Expressed in the Gastric Gland Mucous Cell-type Mucin

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