# Mucin Synthesis. UDP-GlcNAc:GalNAc-R β3-*N*-Acetylglucosaminyltransferase and UDP-GlcNAc:GlcNAcβ1–3GalNAc-R (GlcNAc to GalNAc) β6-*N*-Acetylglucosaminyltransferase from Pig and Rat Colon Mucosa†

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ABSTRACT: Pig and rat colon mucosal membrane preparations catalyze the in vitro transfer of *N*-acetyl-D-glucosamine (GlcNAc) from UDP-GlcNAc to GalNAc-ovine submaxillary mucin to form GlcNAcβ1–3GalNAc-mucin. Rat colon also catalyzes the in vitro transfer of GlcNAc from UDP-GlcNAc to GlcNAcβ1–3GalNAc-mucin to form GlcNAcβ1–3(GlcNAcβ1–6)GalNAc-mucin. This is the first demonstration of in vitro synthesis of the GlcNAcβ1–3GalNAc disaccharide and of the GlcNAcβ1–3-(GlcNAcβ1–6)GalNAc trisaccharide, two of the four major core types found in mammalian glycoproteins of the mucin type, i.e., those containing oligosaccharides with GalNAc-α-serine (threonine) linkages. The activity catalyzing synthesis of the disaccharide has been named UDP-GlcNAc:GalNAc-R β3-*N*-acetylglucosaminyltransferase (mucin core 3 β3-GlcNAc-transferase), while the activity responsible for synthesizing the trisaccharide has been named UDP-GlcNAc:GlcNAcβ1–3GalNAc-R (GlcNAc to GalNAc) β6-*N*-acetylglucosaminyltransferase (mucin core 4 β6-GlcNAc-transferase). The β3-GlcNAc-transferase from pig colon is activated by Triton X-100, has an absolute requirement for $Mn^{2+}$, and transfers GlcNAc to GalNAc-α-phenyl, GalNAc-α-benzyl, and GalNAc-ovine submaxillary mucin with apparent $K_m$ values of 5, 2, and 3 mM and $V_{max}$ values of 59, 62, and 37 nmol $h^{-1}$ (mg of protein)$^{-1}$, respectively. The rat colon β6-GlcNAc-transferase is active in the absence of detergent, is stimulated 17% by addition of detergent, does not require addition of divalent cation, and transfers GlcNAc to GlcNAcβ1–3GalNAc-α-benzyl with an apparent $K_m$ of 0.6 mM and a $V_{max}$ of 714 nmol $h^{-1}$ (mg of protein)$^{-1}$. Mucin products were prepared on a large scale (0.26–0.43 μmol) and were treated with alkaline borohydride to release oligosaccharides; these were analyzed by gel filtration and high-performance liquid chromatography. The pig colon preparation produced a single radioactive oligosaccharide product, identified as GlcNAcβ1–3GalNAcOH, while the rat colon preparation formed two radioactive oligosaccharide products in a 1:1 ratio, identified as GlcNAcβ1–3GalNAcOH and GlcNAcβ1–3(GlcNAcβ1–6)GalNAcOH. Products were identified by proton nuclear magnetic resonance spectroscopy and methylation analysis. The β3-GlcNAc-transferase is therefore present in pig and rat colon and was also detected in rat, human, and monkey colon. Low levels were found in pig, rat, monkey, and sheep stomach. There was no detectable activity in pig, rat, and dog submaxillary gland. The β6-GlcNAc-transferase is present in rat colon and was also detected in pig, human, and monkey colon, in pig, rat, monkey, and sheep stomach, and in rat and dog submaxillary gland. It thus has a wider distribution than the β3-GlcNAc-transferase. Competition experiments suggest that, in the rat colon mucosa, core 4 β6-GlcNAc-transferase may be the same enzyme as UDP-GlcNAc:Galβ1–3GalNAc-R (GlcNAc to GalNAc) β6-GlcNAc-transferase, the enzyme responsible for core 2 synthesis.

GalNAc-α-serine[1] (threonine) linked oligosaccharides with more than two sugar residues have been classified into four core classes: (1) Galβ1–3GalNAc, (2) Galβ1–3(GlcNAcβ1–6)GalNAc, (3) GlcNAcβ1–3GalNAc, and (4) GlcNAcβ1–3(GlcNAcβ1–6)GalNAc (Schachter & Williams, 1982). These oligosaccharides occur in epithelial mucins, in secreted glycoproteins (e.g., various plasma coagulation factors, bovine fetuin, some immunoglobulins, human chorionic gonadotrophin, and fish antifreeze glycoproteins), and in membrane-bound glycoproteins (e.g., erythrocyte glycophorins from several species) (Sadler, 1984).

We have previously demonstrated in vitro synthesis of core class 1 by UDP-Gal:GalNAc-R β3-Gal-transferase from pig submaxillary glands (Schachter et al., 1971; Schachter & McGuire, 1968) and of core class 2 by UDP-GlcNAc:Galβ1–3GalNAc-R (GlcNAc to GalNac) β6-GlcNAc-transferase from dog submaxillary glands (Williams & Schachter, 1980; Williams et al., 1980). We have also shown that pig gastric mucosal preparations contain UDP-GlcNAc:Galβ1–3($R_1$)GalNAc-$R_2$ (GlcNAc to Gal) β3-GlcNAc-transferase which elongates both core 1 and core 2

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[1] Abbreviations: Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; GalNAcOH, *N*-acetyl-D-galactosaminitol; OSM, ovine submaxillary mucin; MES, 2-(*N*-morpholino)-ethanesulfonic acid; NMR, nuclear magnetic resonance; ACS, aqueous counting scintillant; OCS, organic counting scintillant; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid.

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by incorporation of GlcNAc in β1–3 linkage to Gal (Brockhausen et al., 1983a,b, 1984).

In a preliminary report (Brockhausen et al., 1983a), we showed that rat colon mucosal membrane preparations contained a core 3 β3-GlcNAc-transferase which catalyzed the transfer of GlcNAc from UDP-GlcNAc to GalNAc-α-benzyl to form GlcNAcβ1–3GalNAc-α-benzyl (core class 3). The latter product, however, appeared to be rapidly converted to GlcNAcβ1–3(GlcNAcβ1–6)GalNAc-α-benzyl (core 4), indicating the presence in rat colon mucosa of a highly active core 4 β6-GlcNAc-transferase. A tissue and species survey revealed that pig colon mucosal preparations contained appreciable levels of the core 3 β3-GlcNAc-transferase but relatively little of the interfering core 4 β6-GlcNAc-transferase. In the present study, therefore, the kinetic properties and substrate specificities of the core 3 β3-GlcNAc-transferase and core 4 β6-GlcNAc-transferase are studied in pig colon and rat colon, respectively.

## Experimental Procedures

*Materials.* The following materials were purchased from commercial sources: OCS and ACS from Amersham; CMP-*N*-acetyl[4-$^{14}$C]neuraminic acid (1.6 mCi/mmol) and [1-$^{14}$C]acetic anhydride (10.0 mCi/mmol) from New England Nuclear; UDP-GlcNAc, GalNAc, $NaBH_4$, α-D-glucosamine 1-phosphate, uridine 5′-monophosphomorpholidate (4-morpholine–*N*,*N*′-dicyclohexylcarboxamidine salt) and Triton X-100 from Sigma; GalNAc-α-phenyl from Koch-Light Labs; 2-(*N*-morpholino)ethanesulfonate (MES) from Calbiochem-Behring; Bio-Gel P-2 (200–400 mesh), Bio-Gel P-4 (–400 mesh), AG 1-X8 (100–200 mesh), AG 1-X2 (100–200 mesh) and AG 50W-X2 ($H^+$, 200–400 mesh) from Bio-Rad; Sephadex G-25 (fine) from Pharmacia; acetonitrile (UV grade) from Caledon Laboratories.

UDP-*N*-[1-$^{14}$C]acetyl-D-glucosamine (5 mCi/mmol) was synthesized as previously described (Roseman et al., 1961) by acetylating glucosamine 1-phosphate with [1-$^{14}$C]acetic anhydride and coupling *N*-[1-$^{14}$C]acetylglucosamine 1-phosphate with UMP-morpholidate in anhydrous pyridine. Galβ1–3GalNAc (Flowers & Shapiro, 1965), GlcNAcβ1–3(GlcNAcβ1–6)GalNAc-α-benzyl (Abbas et al., 1983a), GlcNAcβ1–3GalNAc-α-benzyl (Abbas et al., 1983b), and Galβ1–3(GlcNAcβ1–6)GalNAc, GalNAc-α-phenyl, GalNAc-α-benzyl, and Galβ1–3GalNAc-α-benzyl (K. L. Matta, unpublished results) were synthesized by methods described elsewhere. *N*-Acetyl-D-galactosaminitol (GalNAcOH), Galβ1–3GalNAcOH, and Galβ1–3(GlcNAcβ1–6)GalNAcOH were prepared respectively from GalNAc and the corresponding reducing oligosaccharides by reduction at room temperature for 4 h with 1 M $NaBH_4$ at pH 10. The reduced compounds were then acidified with AG 50W-X2 ($H^+$), repeatedly flash evaporated from methanol to remove methyl borate, and purified by gel filtration on Bio-Gel P-2 or P-4 equilibrated in water. The purity of these preparations was verified by proton nuclear magnetic resonance (NMR) spectroscopy at 360 MHz. Bovine testicular β-galactosidase (Distler & Jourdian, 1978) was a kind gift from Dr. G. W. Jourdian, Ann Arbor, MI.

*Enzyme Preparations.* Postcaecum colons from freshly slaughtered pigs and colons from freshly killed rats were cleaned with water, the overlying mucus was removed with a glass slide, and the mucosa was scraped off with a scalpel blade. Microsome suspensions were prepared as previously described for pig gastric mucosa (Brockhausen et al., 1983b) by homogenization of tissues in ice-cold 0.25 M sucrose–0.2 M NaCl and centrifugaton at 10000 rpm (Beckman JA17 rotor) for 10 min followed by centrifugation of the supernatant at 140000*g* for 20 min to produce a microsomal pellet. The pellets were suspended in minimal amounts of 0.25 M sucrose and stored at −70 °C. Tissue and species survey was carried out on 0.25 M sucrose homogenates of tissues from freshly killed animals or from human patients after surgery. Gastric mucosa preparations were obtained as previously described (Brockhausen et al., 1983b). Human colon was healthy tissue from cancer patients, who had undergone colon resection.

*Analytical Methods.* Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. Amino sugars were determined on a Durrum D500 amino acid analyzer (Brockhausen et al., 1983b). Liquid scintillation counting was carried out with ACS or OCS by using an LKB Rackbeta scintillation counter. Gas–liquid chromatography was carried out as previously described (Williams & Schachter, 1980).

*High-Performance Liquid Chromatography (HPLC).* HPLC was carried out on a Waters system equipped with a Model 6000A solvent delivery system, Model 510 pumps, Model 680 automated gradient controller, Model 730 data module, and a Model U6K injector. Reduced oligosaccharides were run at 0.7 mL/min on a 5-μ Alltech amino column (250 × 4.6 mm) protected with a CSK guard column packed with ODS (Whatman) under 800–900 psi of pressure. Acetonitrile and deionized water (67:33) were used as the mobile phase at room temperature. Phenyl glycosides were separated at 1 mL/min on a Whatman Partisil PXS 5/25 PAC column protected with a CSK guard column packed with ODS by using isocratic elution with acetonitrile–water (9:1, v/v) for 24 min and linear gradient elution for the next 15 min to an acetonitrile–water ratio of 83:17 (v/v) followed by isocratic elution with acetonitrile–water (83:17 v/v). Standard compounds were analyzed daily. Chromatographic separations were monitored by absorption at 195 nm with a Waters Lambda-Max Model 480 LC spectrophotometer. The elution of radioactive compounds was determined by collecting fractions and counting suitable aliquots. The reproducibility of retention times from experiment to experiment was routinely over 90%.

*Proton Nuclear Magnetic Resonance (NMR) Spectroscopy.* Samples were prepared for NMR as previously described (Brockhausen et al., 1983a,b) except that the final preparation was dissolved in 99.996% $D_2O$ (Aldrich) and treatment with Chelex-100 was omitted. Spectra were recorded with a Nicolet 360-MHz spectrometer (Carver & Grey, 1981; Longmore & Schachter, 1982).

*Methylation Analysis.* Samples (about 50 nmol) were dried and subjected to methylation analysis as previously described (Longmore & Schachter, 1982) except that hydrolysis of the permethylated oligosaccharide was carried out with 95% acetic acid–0.5 N $H_2SO_4$ at 80 °C overnight (Stellner et al., 1973) instead of with trifluoroacetic acid. The hydrolysate was neutralized with sodium hydroxide, reduced with $NaBD_4$, reacetylated (pyridine–acetic anhydride, 1:1, at room temperature for 20 h), and subjected to analysis with a Hewlett-Packard 5985B gas chromatograph–quadrupole mass spectrometer operating in the electron impact mode and coupled to a Hewlett-Packard HP-1000E series computer. Basic and neutral residues were analyzed in a single run by using a SP-2100 fused silica-capillary column (25 m) and helium carrier gas at a flow rate of 10 mL/min. The temperature program was from 120 to 280 °C at 4 or 8 °C/min with a 6-min delay at 120 °C prior to the temperature program.

*Preparation of GalNAc-α-OSM.* Ovine submaxillary mucin (OSM) contains mainly sialylα2–6GalNAc but also contains small amounts of Galβ1–3GalNAc (less than 4%) which could act as an acceptor for GlcNAc-tranferases other than the core 3 enzyme. OSM containing 154 μmol of GalNAc was therefore subjected to mild acid hydrolysis with 0.5 N HCl at 80 °C for 1 h under nitrogen to remove sialic acid, fucose, and some of the Gal bound to GalNAc. The hydrolysate was neutralized with NaOH and dialyzed against water and the mucin recovered by lyophilization. The mucin was then incubated with 130 milliunits of bovine testicular β-galactosidase in citrate–phosphate buffer, pH 4.3, at 37 °C for 18 h as previously described (Brockhausen et al., 1983b). Galactosidase was inactivated at 80 °C fo 10 min, and the mucin was purified on a Sephadex G-25 column (2.4 × 93 cm) equilibrated in water. Gas–liquid chromatography of the product (GalNAc-OSM) indicated that fucose, Gal, GlcNAc, and sialic acid constituted less than 0.5% relative to GalNAc content.

*GlcNAc-transferase Assays.* The standard assay contained 0.1 M MES, pH 7, 0.2% (v/v) Triton X-100, 10 mM $MnCl_2$, and 2.8 mM UDP-*N*-[1-$^{14}$C]acetylglucosamine (300–1700 dpm/nmol) in a total volume of 0.050 mL. The β3-GlcNAc-transferase was assayed with 4 mM GalNAc-α-phenyl and pig colon mucosal microsomes (0.3 mg of protein) while the β6-GlcNAc-transferase was assayed with 0.5 mM GlcNAcβ1–3GalNAc-α-benzyl and rat colon mucosal microsomes (0.08–0.16 mg of protein). After incubation at 37 °C for 30 min, 0.4 mL of 20 mM sodium tetraborate–1 mM EDTA (pH 9.1) was added to stop the reaction, and the mixture was passed through a small column (Pasteur pipet) containing about 1 mL of AG 1-X2 (100–200 mesh) or AG 1–X8 (100–200 mesh), in the chloride form. The columns were washed with 2.6 mL of water, and the total eluate was added to 17 mL of ACS and counted.

Assays for the β3-GlcNAc-transferase were also carried out as above with GalNAc-OSM (180 nmol of GalNAc) as acceptor instead of GalNAc-α-phenyl. The reaction was stopped by adding 0.010 mL of 1% sodium tetraborate–0.25 M EDTA, and the product was isolated by high-voltage electrophoresis on Whatman 3MM paper in 1% sodium tetraborate for 1 h at 2 kV and 160 mA followed by descending paper chromatography with 80% ethyl alcohol overnight. The areas containing product were cut out and counted.

Control incubations without acceptor were carried out routinely, and the endogenous incorporation was subtracted in calculation of enzyme rates.

*Sialyltransferase Assays.* The incubations contained 0.05 M MES, pH 6, 0.5% Triton X-100, 0.5 mM CMP-*N*-acetyl[4-$^{14}$C]neuraminic acid (3520 dpm/nmol), 2 mM acceptor, and either pig colon microsomes (0.32 mg of protein) or rat colon homogenate (0.22 mg of protein) in a final volume of 0.050 mL. After 1 h at 37 °C, the reaction was stopped by freezing, and the product was separated from nucleotide–sugar and free sialic acid by high-voltage paper electrophoresis in 1% sodium tetraborate for 1 h at 2 kV and 160 mA. Radioactive product was located and counted by cutting the paper into 1-in. strips and counting each strip in OCS.

*Large-Scale Preparation of Product Formed with Pig Colon Enzyme.* GalNAc-OSM (36 μmol of GalNAc) was incubated in a total volume of 5 mL containing 0.1 M MES, pH 6.5, 10 mM $MnCl_2$, 0.1% Triton X-100, 2.8 mM UDP-*N*-[1-$^{14}$C]acetylglucosamine (420 dpm/nmol), and pig colon microsomes (32 mg of protein). After incubation at 37 °C for 3 h, the mixture was centrifuged at 2000 rpm for 15 min, and the pellet was washed twice with water. The combined supernatants were lyophilized, and the mucin product was purified on Sephadex G-25 (1.6 × 38 cm) equilibrated with 0.1 N acetic acid. Oligosaccharides were released as alditols by incubation at 45 °C for 18 h in 40 mL of 0.05 M KOH–1 M $NaBH_4$. The pH of the incubation was lowered to 5 by addition of acetic acid, and the mixture was passed through a column (2.4 × 40 cm) of AG 50-X2 in the hydrogen form. Borate was removed by flash evaporation from methanol. Oligosaccharide alditols were purified on a Bio-Gel P-4 column (–400 mesh; 1.6 × 90 cm) equilibrated in water followed by HPLC.

*Large-Scale Preparation of Product Formed with Rat Colon Enzyme.* GalNAc-OSM (54 μmol of GalNAc) was incubated in a total volume of 7.5 mL containing 0.1 M MES, pH 6.5, 5 mM $MnCl_2$, 0.1% Triton X-100, 2.8 mM UDP-*N*-[1-$^{14}$C]acetylglucosamine (420 dpm/nmol), rat colon mucosal microsomes (14 mg of protein) and rat colon mucosal homogenate (14 mg of protein). After incubation at 37 °C for 3 h, the mixture was centrifuged at 2000 rpm for 15 min, and the pellet was washed twice with water. The combined supernatants were lyophilized, and the mucin product was purified on Sephadex G-25 (1.6 × 38 cm) equilibrated in 0.1 N acetic acid. Oligosaccharides were released as alditols by incubation at 45 °C for 18 h in 60 mL of 0.05 N KOH–1 M $NaBH_4$ and purified as above for the pig colon enzyme.

## Results

*Pig Colon as a Suitable Tissue for the Study of Core 3 Synthesis.* GlcNAc-transferase incubations were carried out with pig colon mucosal microsomes and GalNAc-α-phenyl as acceptor for various times from 20 to 120 min. The incubations were passed through AG 1-X2 as described under Experimental Procedures, and the eluates were lyophilized and analyzed by HPLC. Three radioactive products were detected by HPLC analysis after 1 h (Figure 1). Peak I was free [$^{14}$C]GlcNAc derived from breakdown of UDP-GlcNAc, peak II eluted in the disaccharide region, and peak III eluted in the trisaccharide region. Peak II was identified as [$^{14}$C]-GlcNAcβ1–3GalNAc-α-phenyl and peak III as [$^{14}$C]-GlcNAcβ1–3([$^{14}$C]GlcNAcβ1–6)GalNAc-α-phenyl on the basis of their HPLC behavior. The formation of peak III by pig colon microsomes reached a maximum of 4% of total products at 1 h. Thus, at least 96% of the radioactive product formed was due to the action of core 3 β3-GlcNAc-transferase. Pig colon mucosa is therefore a suitable tissue for the study of the β3-GlcNAc-transferase since it has relatively low levels of the core 4 β6-GlcNAc-transferase.

*Optimum Assay Conditions for the β3-GlcNAc-transferase.* Under standard assay conditions, incorporation of GlcNAc into either GalNAc-α-phenyl or GalNAc-OSM is proportional to incubation time for at least 30 min. Triton X-100 stimulated enzyme activity with a maximum at 0.1% (v/v) and was inhibitory at concentrations above 0.5%. The enzyme showed a broad pH optimum centering at about 6.5. $Mn^{2+}$ also showed a broad optimum at about 10–20 mM. $Mn^{2+}$ was essential for activity (Table I) and could not be replaced by $Co^{2+}$, $Mg^{2+}$, $Ca^{2+}$, or $Zn^{2+}$. In fact, $Co^{2+}$ inhibited enzyme activity in the presence of $Mn^{2+}$. The activity was not affected by 1 mM mercaptoethanol or 0.2 M GlcNAc.

GalNAc-α-phenyl, GalNAc-α-benzyl, and GalNAc-OSM all showed Michaelis–Menten kinetics under standard conditions with apparent $K_m$ values of 5, 2, and 3.2 mM and $V_{max}$ values of 59, 63, and 37 nmol $h^{-1}$ (mg of protein)$^{-1}$, respectively. Native OSM, containing mainly sialylα2–6GalNAc groups, was ineffective as an acceptor.

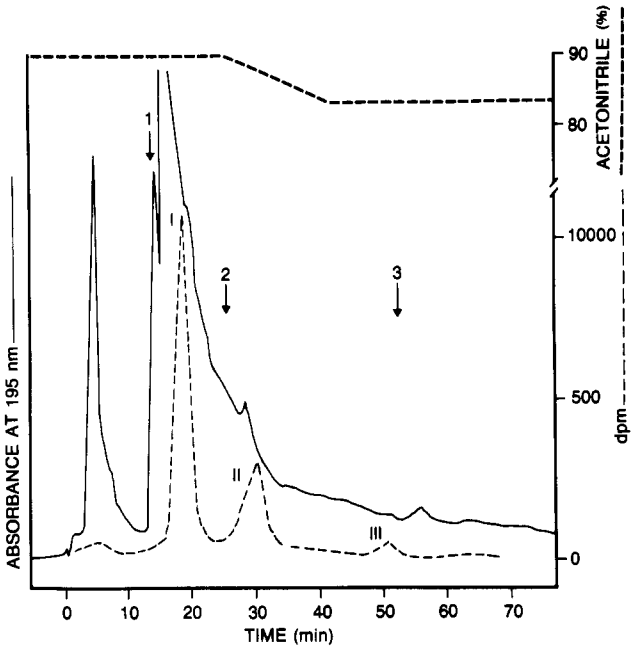


FIGURE 1: HPLC analysis of the products formed after a 1-h incubation under standard conditions using pig colon microsomes as enzyme source and GalNAc-α-phenyl as acceptor. The column was a PAC column eluted at 1 mL/min. The acetonitrile–water ratios are indicated in the figure (---). The elution positions of standards are indicated by arrows: (1) GlcNAc, (2) GlcNAcβ1–3GalNAc-α-benzyl, and (3) GlcNAcβ1–3(GlcNAcβ1–6)GalNAc-α-benzyl. Benzyl glycosides were used as standards because the phenyl glycosides were not available, and we have previously shown (Brockhausen et al., 1983a) that phenyl and benzyl glycosides have very similar elution positions. Three radioactive peaks were detected (---): (I) free [$^{14}$C]GlcNAc, (II) a phenyl disaccharide which is probably [$^{14}$C]-GlcNAcβ1–3GalNAc-α-phenyl, and (III) a phenyl trisaccharide which is probably [$^{14}$C]GlcNAcβ1–3([$^{14}$C]GlcNAcβ1–6)GalNAc-α-phenyl. Absorbance at 195 nm is indicated (—).

Table I: Properties of the Core 3 β3-GlcNAc-transferase[a]

| assay conditions | enzyme activity (%) |
|---|---|
| complete system | 100 |
| minus $Mn^{2+}$ | 0 |
| minus $Mn^{2+}$, plus EDTA | 0 |
| minus $Mn^{2+}$, plus $Co^{2+}$ | 4 |
| minus $Mn^{2+}$, plus $Mg^{2+}$ | 0 |
| minus $Mn^{2+}$, plus $Ca^{2+}$ | 3.5 |
| minus $Mn^{2+}$, plus $Zn^{2+}$ | 2 |
| plus $Co^{2+}$ | 27.5 |

[a] Pig colon mucosal microsomes were assayed in duplicate under standard conditions with GalNAc-α-phenyl as acceptor, and average rates are reported above. EDTA and other cations were added at concentrations of 10 mM. The activity with the complete system was 20 nmol/h$^{-1}$ (mg of protein)$^{-1}$.

*Optimum Assay Conditions for the β6-GlcNAc-transferase.* The acceptor used in the standard assay was GlcNAcβ1–3GalNAc-α-benzyl. When this acceptor is incubated with rat colon mucosal preparations and UDP-GlcNAc, the product formed comigrates on HPLC with a synthetic standard of GlcNAcβ1–3(GlcNAcβ1–6)GalNAc-α-benzyl (Brockhausen et al., 1983a). Under standard assay conditions, incorporation of GlcNAc into GlcNAcβ1–3GalNAc-α-benzyl is proportional to incubation time for at least 30 min and is proportional to protein concentration to at least 0.16 mg of enzyme protein. Triton X-100 stimulated enzyme activity with a maximum at 0.1% (v/v) although there was 85% of maximal activity in the absence of detergent. The enzyme showed a pH optimum at about 6.5. An absolute requirement for exogenous divalent cation could not be demonstrated (Table II). Omission of $Mn^{2+}$ had no effect on activity, and EDTA (10 mM) caused only 24% inhibition. In the absence of $Mn^{2+}$, $Zn^{2+}$ and $Co^{2+}$ inhibited the enzyme while $Mg^{2+}$ and $Ca^{2+}$ had little effect. $Zn^{2+}$ was inhibitory even in the presence of $Mn^{2+}$. AMP (10 mM), ATP (9 mM), and GlcNAc (0.2 M) caused slight stimulation while mercaptoethanol (0.9 mM) caused slight inhibition (data not shown). Native OSM, containing mainly sialylα2–6GalNAc groups, is not a GlcNAc accepter under standard assay conditions.

Table II: Cation Requirements of the Rat Colon Mucosal Core 2 and Core 4 β6-*N*-Acetylglucosaminyltransferases[a]

| assay conditions | enzyme activities (%) | |
|---|---|---|
| | Galβ1–3GalNAc-Bz[b] | GlcNAcβ1–3GalNAcBz[c] |
| complete system | 100 | 100 |
| minus $Mn^{2+}$ | 100 | 100 |
| minus $Mn^{2+}$, plus EDTA | 76 | 76 |
| minus $Mn^{2+}$, plus $Co^{2+}$ | 36 | 27 |
| minus $Mn^{2+}$, plus $Mg^{2+}$ | 80 | 81 |
| minus $Mn^{2+}$, plus $Ca^{2+}$ | 72 | 96 |
| minus $Mn^{2+}$, plus $Zn^{2+}$ | 0 | 0 |
| plus $Zn^{2+}$ | 0 | 0 |

[a] Rat colon mucosal microsomes were assayed in duplicate under standard conditions with 0.5 mM either of Galβ1–3GalNAc-α-benzyl or GlcNAcβ1–3GalNAc-α-benzyl as acceptor, and average rates are reported above. EDTA and other cations were added at concentrations of 10 mM. The actual specific activities for rat colon mucosal microsomes are shown in Table V. [b] Substrate for the core 2 transferase; Bz = α-benzyl. [c] Substrate for the core 4 transferase; Bz = α-benzyl.

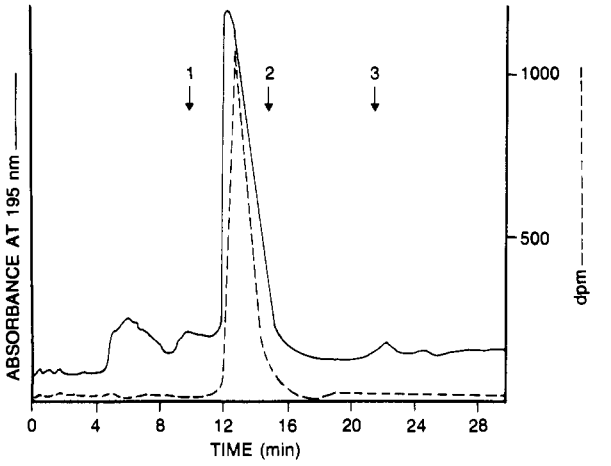


FIGURE 2: HPLC analysis of the oligosaccharide alditol products released by alkaline borohydride treatment of the product formed by the action of pig colon microsomes on GalNAc-OSM. An amino column was used at a flow rate of 0.7 mL/min; the solvent was acetonitrile–water (67:33). The elution positions of standards are indicated by arrows: (1) GalNAcOH, (2) Galβ1–3GalNAcOH, and (3) Galβ1–3(GlcNAcβ1–6)GalNAcOH. The radioactive product (---) was identified as [$^{14}$C]GlcNAcβ1–3GalNAcOH. Absorbance at 195 nm is indicated (—).

*Product Identification for the Pig Colon Enzyme.* A large-scale incubation containing the pig colon mucosal preparation and 36 μmol of GalNAc-OSM yielded 0.43 μmol of product (1.2% conversion). After alkaline borohydride treatment, a single radioactive product peak was detected on Bio-Gel P-4 (data not shown). This product was further purified by preparative HPLC, eluting at 12.9 min, near Galβ1–3GalNAcOH (Figure 2). No radioactivity was detected in the trisaccharide region (Figure 2).

Table III shows the chemical shifts for this product after NMR spectroscopy at 360 MHz at 70 °C and room temperature. The spectrum at room temperature is identical with the spectrum reported for GlcNAcβ1–3GalNAcOH by Van Halbeek et al. (1982). The doublet at 4.604 ppm (room

Table III: Proton Chemical Shifts (ppm) Derived from High-Resolution Proton NMR Spectroscopy[a]

| protons | pig colon oligosaccharide alditol product at 70 °C | pig colon oligosaccharide alditol product at 18 °C | GlcNAcβ1-3GalNAc-OH[b] at 25 °C |
|---|---|---|---|
| GalNAcOH | | | |
| H-2 | 4.274 | 4.288 | 4.287 |
| H-3 | 3.989 | 3.999 | 3.996 |
| H-4 | ND | 3.542 | 3.546 |
| H-5 | 4.103 | 4.146 | 4.141 |
| H-6 | ND | ND | 3.65 |
| *N*-acetyl | 2.039 | 2.038 | 2.037 |
| GlcNAcβ1-3 | | | |
| H-1 | 4.612 (8.4) | 4.604 (8.5) | 4.604 |
| H-6 | 3.944 | 3.948 | 3.950 |
| *N*-acetyl | 2.083 | 2.086 | 2.085 |

[a] Numbers in parentheses refer to coupling constants in hertz. ND, not determined. [b] Data from Van Halbeek et al. (1982).

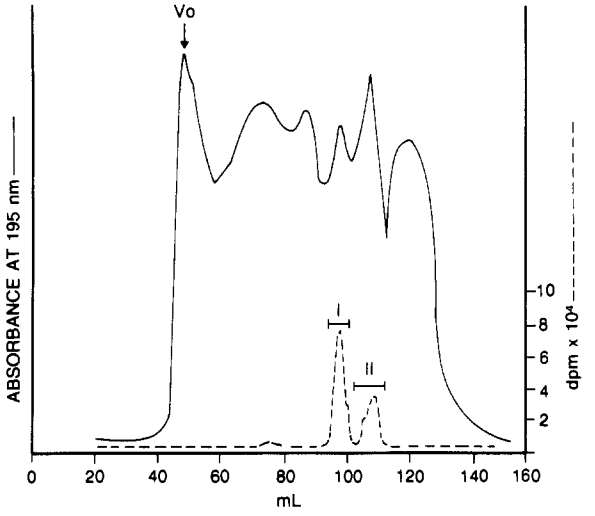


FIGURE 3: Bio-Gel P-4 column (1.6 × 90 cm) chromatography of reduced oligosaccharides released by alkaline borohydride from the product of rat colon mucosal microsome action on GalNAc-OSM. Two radioactive peaks (I and II) are resolved (---). Absorbance at 195 nm is indicated (—).

temperature) is characteristic of the anomeric hydrogen of GlcNAc linked β1–3 to GalNAcOH and can be distinguished from the signals for GlcNAc linked β1–3 to Gal [4.66–4.68 ppm (Brockhausen et al., 1984)] and β1–6 to GalNAcOH [4.535 ppm (Brockhausen et al., 1984)], neither of which were detected. The H-5 signal of GalNAcOH at 4.146 ppm indicates that C-6 of GalNAcOH is not substituted, and the signals for H-2 and H-3 of GalNAcOH are diagnostic of the core 3 structure (Van Halbeek et al., 1982).

Methylation analysis of the oligosaccharide alditol product indicated the presence of 3,4,6-tri-*O*-methyl-1,5-di-*O*-acetyl-2-deoxy-2-(*N*-methylacetamido)hexitol due to a terminal GlcNAc residue and 1,4,5,6-tetra-*O*-methyl-3-*O*-acetyl-2-deoxy-2-(*N*-methylacetamido)hexitol due to a 3-substituted GalNAcOH residue. These derivatives were identified by their characteristic elution times (14.4 and 13.0 min, respectively) and mass spectra. No other methylated derivatives were detected.

It is concluded that the only product formed in the large-scale incubation was [$^{14}$C]GlcNAcβ1–3GalNAc-OSM.

*Product Identification for the Rat Colon Enzyme.* After a large-scale incubation containing the rat colon mucosal preparation and 54 μmol of GalNAc-OSM, 0.78 μmol of GlcNAc was incorporated into mucin. On alkaline borohydride treatment, two radioactive oligosaccharide alditols were detected on Bio-Gel P-4 (Figure 3), peaks I (0.26 μmol) and II (0.27 μmol). These fractions were further purified by preparative HPLC. Peak II migrated on HPLC at the same position as a marker of [$^{14}$C]GlcNAcβ1–3GalNAcOH (data not shown); the latter disaccharide was prepared (see above) by the action of pig colon β3-GlcNAc-transferase on GalNAc-OSM. Peak I eluted on HPLC in the trisaccharide region just ahead of a marker of Galβ1–3(GlcNAcβ1–6)-GalNAcOH (Figure 4).

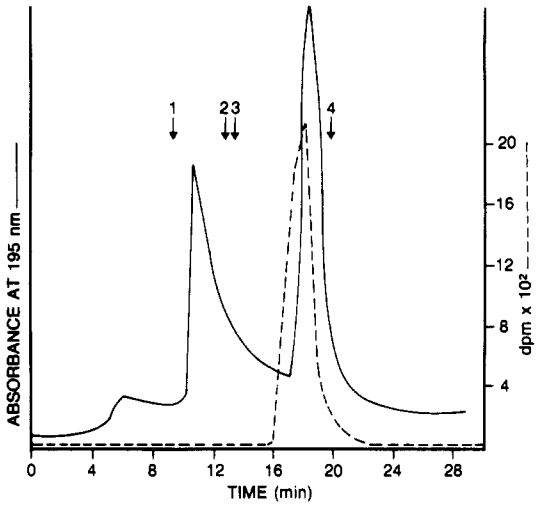


FIGURE 4: HPLC chromatography of peak I obtained from the Bio-Gel P-4 column (Figure 3) on a 5-μ Alltech amino column (250 × 4.6 mm) at 800–900 psi of pressure and 0.7 mL/min with acetonitrile–water (67:33) as the mobile phase. The elution positions of the following standards are indicated by arrows: (1) GalNAcOH, (2) [$^{14}$C]GlcNAcβ1–3GalNAcOH, (3) Galβ1–3GalNAcOH, and (4) Galβ1–3(GlcNAcβ1–6)GalNAcOH. Absorbance at 195 nm (—) and dpm (---) are indicated.

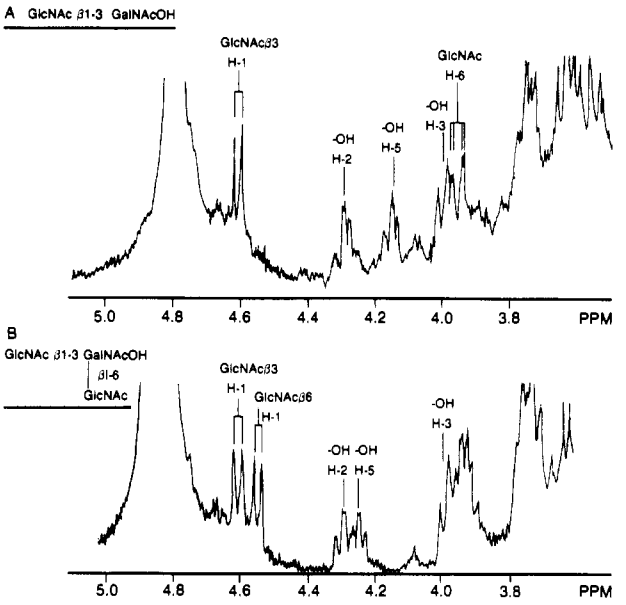


FIGURE 5: 360-MHz NMR spectra at room temperature between 3.8 and 5.0 ppm of [$^{14}$C]GlcNAcβ1–3GalNAcOH and [$^{14}$C]-GlcNAcβ1–3([$^{14}$C]GlcNAcβ1–6)GalNAcOH, reduced oligosaccharides released from the products formed by the action of rat colon mucosal microsomes on GalNAc-OSM.

Figure 5 shows the high-resolution NMR spectra for these two products, at room temperature, between 3.8 and 5.0 ppm. The chemical shifts at both room temperature and 70 °C are summarized in Table IV. The spectra for peak II are identical with the spectra for GlcNAcβ1–3GalNAcOH shown in Table III and reported by Van Halbeek et al. (1982). No GlcNAcβ1–6GalNAcOH was detected.

Table IV: Proton Chemical Shifts (ppm) Derived from High-Resolution Proton NMR Spectroscopy[a]

| protons | rat colon enzyme–oligosaccharide alditol products: peak II at 70 °C | peak II at 19 °C | peak I at 70 °C | peak I at 19 °C | GlcNAcβ1-3GalNAcOH[b] / GlcNAcβ1-6 / Galβ1-4 at 25 °C |
|---|---|---|---|---|---|
| GalNAcOH | | | | | |
| H-2 | ND | 4.286 | ND | 4.282 | 4.282 |
| H-3 | 3.988 | 3.998 | 3.978 | 3.985 | 3.986 |
| H-4 | ND | 3.541 | ND | ND | 3.515 |
| H-5 | 4.099 | 4.144 | 4.199 | 4.236 | 4.239 |
| H-6 | ND | ND | ND | ND | 3.906 |
| *N*-acetyl | 2.039 | 2.037 | 2.045 | 2.045 | 2.045 |
| GlcNAcβ1–3 | | | | | |
| H-1 | 4.612 (8.4) | 4.604 (8.3) | 4.607 (8.1) | 4.598 (8.3) | 4.599 |
| *N*-acetyl | 2.083 | 2.085 | 2.080 | 2.081 | 2.081 |
| GlcNAcβ1–6 | | | | | |
| H-1 | | | 4.562 (8.1) | 4.539 (8.4) | 4.564 |
| *N*-acetyl | | | 2.062 | 2.063 | 2.061 |

[a] Numbers in parentheses refer to coupling constants in hertz. ND, not determined. [b] Data from Van Halbeek et al. (1982).

The peak I spectrum (Figure 5B) differs from the peak II spectrum (Figure 5A) by having an additional doublet at 4.539 ppm (room temperature) due to the anomeric hydrogen of a β6-linked GlcNAc residue and by a marked downfield shift of the GalNAcOH H-5 signal from 4.144 to 4.236 ppm, indicating substitution of carbon 6 of GalNAcOH (Van Halbeek et al., 1982). The peak I spectrum is very similar to the spectrum for GlcNAcβ1–3(Galβ1–4GlcNAcβ1–6)GalNAcOH previously reported by Van Halbeek et al. (1982) (Table IV).

Methylation analysis of the peak I oligosaccharide alditol product showed 3,4,6-tri-*O*-methyl-1,5-di-*O*-acetyl-2-deoxy-2-(*N*-methylacetamido)hexitol due to terminal GlcNAc residues and 1,4,5-tri-*O*-methyl-3,6-di-*O*-acetyl-2-deoxy-2-(*N*-methylacetamido)hexitol due to a 3,6-disubstituted GalNAcOH residue. These derivatives were identified by their characteristic elution times (14.5 and 15.1 min, respectively) and mass spectra. No other methylated derivatives were detected.

It is concluded that the only products formed in the large-scale incubations were [$^{14}$C]GlcNAcβ1–3GalNAc-OSM and [$^{14}$C]GlcNAcβ1–3([$^{14}$C]GlcNAcβ1–6)GalNAc-OSM in an approximately 1:1 ratio.

*Comparison of Core 2 and Core 4 β6-GlcNAc-transferases.* Table II compares the cation requirements of the core 4 β6-GlcNAc-transferase with those of the core 2 enzyme, UDP-GlcNAc:Galβ1–3GalNAc-R (GlcNAc to GalNAc) β6-GlcNAc-transferase (Williams & Schachter, 1980; Williams et al., 1980), by using rat colon mucosa as a source for both enzymes. The marked similarity between the two enzymes prompted a competition study (Table V) which indicated that the two substrates Galβ1–3GalNAc-α-benzyl and GlcNAcβ1–3GalNAc-α-benzyl competed for a common active site. The evidence therefore suggests, but does not conclusively prove, that the core 2 and core 4 β6-GlcNAc-transferase activities reside in a single enzyme, at least in the rat colon mucosa.

*Tissue and Species Survey.* Table VI shows a survey for the presence of mucin core 2, core 3, and core 4 GlcNAc-transferases in various tissues and species. Homogenates rather than microsomes were used as enzyme sources in this survey, and definitive product identifications were not carried out.

All colonic tissues tested (pig, rat, monkey, and human) showed appreciable core 3 β3-GlcNAc-transferase activity. Stomach mucosa from four species showed relatively low levels of core 3 activity whereas submaxillary gland from three species showed no detectable enzyme.

In contrast, both the core 2 and core 4 GlcNAc-transferase activities are widely distributed and were detected in all tissues tested except pig submaxillary gland. Control experiments showed the pig submaxillary gland extract to contain UDP-Gal:GalNAc-R β3-galactosyltransferase. The absence of β6-GlcNAc-transferase in pig submaxillary gland was to be expected since pig submaxillary mucin does not contain a GlcNAc linked β1–6 to GalNAc. The variations in the ratio of activities of core 2 enzyme to core 4 enzyme in different

Table V: Mixed Substrate Experiment for Core 2 and Core 4 β6-GlcNAc-transferases[a]

| substrate concentrations (mM): Galβ1–3-GalNAcαBz | GlcNAc-β1–3-GalNAc-αBz | transferase activities [nmol h$^{-1}$ (mg of protein)$^{-1}$]: exptl | calcd for[b]: 1 E | 2 E | conclusion |
|---|---|---|---|---|---|
| 0.5 | | 298 | | | |
| 1.0 | | 523 | | | |
| 2.0 | | 568 | | | |
| 3.0 | | 576 | | | |
| | 0.5 | 304 | | | |
| | 1.0 | 479 | | | |
| | 2.0 | 549 | | | |
| | 3.0 | 559 | | | |
| 0.5 | 0.5 | 491 | 456 | 603 | 1 E |
| 1.0 | 1.0 | 583 | 573 | 1000 | 1 E |
| 2.0 | 2.0 | 607 | 656 | 1120 | 1 E |
| 3.0 | 3.0 | 620 | 689 | 1140 | 1 E |

[a] Rat colon mucosal microsomes (0.14 mg of protein) were incubated for 30 min at 37 °C in a total volume of 0.050 mL containing 0.1 M MES, pH 6.5, 0.1% Triton X-100, 5 mM $MnCl_2$, 2.8 mM UDP-*N*-[1-$^{14}$C]acetylglucosamine (540 dpm/nmol), and Galβ1–3GalNAc-α-benzyl or GlcNAcβ1–3GalNAc-α-benzyl, either separately or together, at the concentrations shown. Product was measured as described under Experimental Procedures. Rates are averages of duplicate determinations. Bz = benzyl. [b] If two enzymes are acting on the two substrates, the amount of radioactivity incorporated into them when incubated in combination is the sum of the radioactivity incorporated into the substrates when incubated separately. The following equation was used to calculate the amount of radioactivity incorporated into the substrates assuming competition for a common enzyme active site (Dixon & Webb, 1964): $v = [V_1(S_1/K_1) + V_2(S_2/K_2)]/(1 + S_1/K_1 + S_2/K_2)$. $S_1$ and $S_2$ are the concentrations of the two substrates, $V_1$ and $V_2$ are the maximal velocities, and $K_1$ and $K_2$ are the apparent $K_m$ values. The $V_{max}$ values for Galβ1–3GalNAc-α-benzyl and GlcNAcβ1–3GalNAc-α-benzyl are, respectively, 840 and 714 nmol h$^{-1}$ (mg of protein)$^{-1}$, and the respective $K_m$ values are 0.8 and 0.6 mM.

Table VI: Tissue and Species Survey for Core 2, Core 3, and Core 4 GlcNAc-transferases (Gn-T)[a]

| enzyme source | transferase activity (nmol $h^{-1}$ $mg^{-1}$) core 3 β3-Gn-T | core 4 β6-Gn-T | core 2 β6-Gn-T | ratio of activities (core 2/ core 4) |
|---|---|---|---|---|
| rat | | | | |
| colon | 19.7 | 108 | 135 | 1.3 |
| stomach | 0.4 | 25.2 | 29.6 | 1.2 |
| submaxillary gland | 0 | 4.1 | 4.4 | 1.1 |
| pig | | | | |
| colon | 20.5 | 13.4 | 51.2 | 3.8 |
| stomach | 0.8 | 167 | 334 | 2.0 |
| submaxillary gland | 0 | 0 | 0 | |
| dog | | | | |
| submaxillary gland | 0 | 17.8 | 114 | 6.4 |
| monkey | | | | |
| colon | 2.4 | 4.7 | 11.9 | 2.5 |
| stomach | 0.4 | 9.5 | 25.9 | 2.7 |
| human | | | | |
| colon | 5.5 | 10.3 | 24.0 | 2.3 |
| sheep | | | | |
| stomach | 0.6 | 16.9 | 21.3 | 1.3 |

[a] Enzyme extracts (0.12–0.66 mg of protein) were incubated for 1 h at 37 °C in a total volume of 0.050 mL containing 0.1 M MES, pH 7, 0.1% Triton X-100, 10 mM $MnCl_2$, 2.8 mM UDP-*N*-[1-$^{14}$C]acetylglucosamine (420–540 dpm/nmol), and 1 mM GalNAc-α-benzyl (core 3 β3-GlcNAc-transferase), 1 mM Galβ1–3GalNAc-α-benzyl (core 2 β6-GlcNAc-transferase), or 1 mM GlcNAcβ1–3GalNAc-α-benzyl (core 4 β6-GlcNAc-transferase). Product was measured as described under Experimental Procedures. Rates are averages of duplicate determinations. Most of the above tissues have been assayed several times with similar results.

tissues is shown in Table VI. Although the number of tissues studied is limited, the data support the hypothesis that a single enzyme is responsible for both core 2 and core 4 activities. It appears further that this enzyme differs between species since the ratio of activities is approximately constant within a single species, being 1.2 for rats and sheep, 2.3–3.8 for pigs, monkeys, and humans, and 6 for the dog. It will be of interest to determine whether these rules hold for other mucin-secreting tissues in these species.

*Sialyltransferase Assays.* Since rat colon mucosa has a highly active core 4 β6-GlcNAc-transferase, it is surprising that rat colonic mucin has oligosaccharides with core class 3 but not core class 4 (Slomiany et al., 1980). One possible explanation is that GlcNAcβ1–3GalNAc-R may be acted on more effectively by another glycosyltransferase, e.g., a sialyltransferase. To test this hypothesis, pig and rat colon mucosal preparations were assayed for sialyltransferase activity toward GlcNAcβ1–3GalNAc-α-benzyl and Galβ1–3GalNAc-α-benzyl. Neither tissue transferred sialic acid to GlcNAcβ1–3GalNAc-α-benzyl. However, Galβ1–3GalNAc-α-benzyl was an excellent sialyltransferase substrate [12.8 and 14.0 nmol $h^{-1}$ (mg of protein)$^{-1}$ for pig and rat colon, respectively].

## Discussion

We have demonstrated the in vitro synthesis of mucin cores 3 and 4 (Figure 6). Both rat and pig colon mucosal microsomes can catalyze the transfer of GlcNAc from UDP-GlcNAc to GalNAc-OSM to form the core 3 structure GlcNAcβ1–3GalNAc-OSM. It is proposed that this enzyme be called UDP-GlcNAc:GalNAc-R β3-GlcNAc-transferase (or core 3 β3-GlcNAc-transferase). The presence in rat colon of a highly active core 4 β6-GlcNAc-transferase prevents proper assay and study of the core 3 enzyme. This interference can be avoided by using pig colon. With GalNAc-α-phenyl as acceptor, more than 96% of the product formed by pig colon

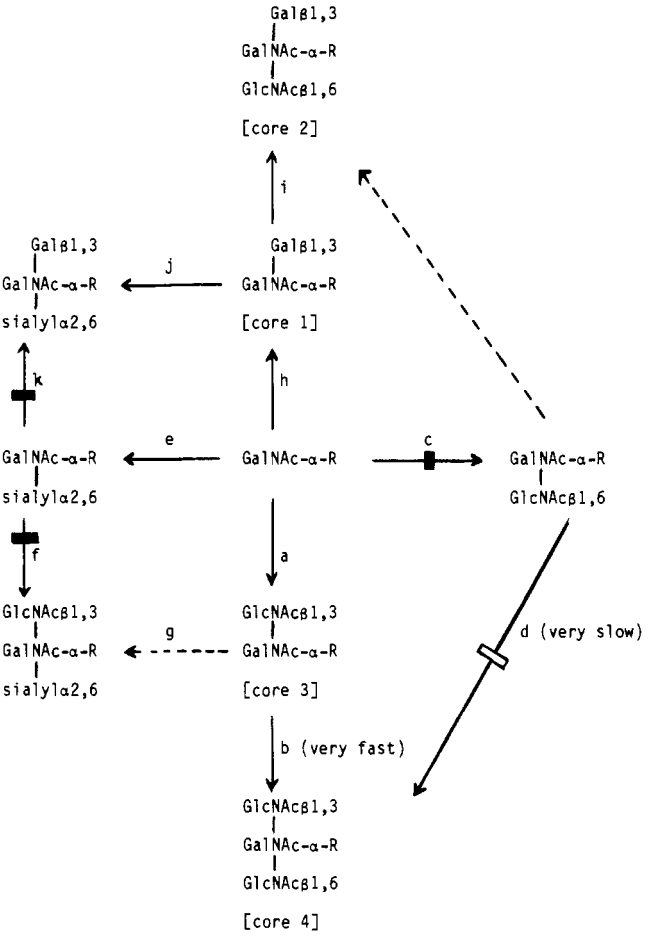


FIGURE 6: Scheme for the biosynthesis of the four major mucin core types. The enzymes for the synthesis of cores 1 and 2 (steps h and i) have been described previously. This paper describes the enzymes involved in assembly of cores 3 and 4 (steps a and b). Reactions shown to either proceed or not proceed in vitro are indicated by continuous arrows; the two reactions shown in discontinuous arrows have not as yet been conclusively studied in vitro.

microsomes was due to core 3 GlcNAc-transferase. With GalNAc-OSM as acceptor, no core 4 product was detected at all.

A tissue and species survey indicated that colon mucosa from four species was enriched in core 3 β3-GlcNAc-transferase. Gastric tissues had relatively low amounts of this enzyme whereas submaxillary gland had no detectable activity.

The core 3 β3-GlcNAc-transferase is present in the microsome fraction, is stimulated by Triton X-100 and, like most glycosyltransferases, is probably a membrane-bound enzyme. The absolute requirement for $Mn^{2+}$ and the inhibitory effect of $Co^{2+}$ distinguishes this enzyme from the elongation β3-GlcNAc-transferase (Brockhausen et al., 1983b, 1984) which adds GlcNAc to Gal. Further, pig gastric mucosa is relatively rich in elongation β3-GlcNAc-transferase (Brockhausen et al., 1984) but has very low core 3 β3-GlcNAc-transferase (Table VI).

The core 3 β3-GlcNAc-transferase can act on GalNAc-α-R where R is phenyl, benzyl, or OSM but not on sialylα2-6GalNAc-OSM. The structure GlcNAcβ1–3(sialylα2–6)-GalNAc-R is found in mucins from rat colon (Slomiany et al., 1980), rat sublingual gland (Slomiany & Slomiany, 1978), bovine submaxillary gland (Tsuji et al., 1982), bonnet monkey cervix (Nasir-ud-Din et al., 1981), and human rectal adenocarcinoma (Kurosaka et al., 1983). The synthetic path for this trisaccharide is probably via steps a and g (Figure 6). GlcNAcβ1–3GalNAc oligosaccharides have also been detected

in mucins from rat small intestine (Carlsson et al., 1978), horse stomach (Newman & Kabat, 1976), and human bronchial tissue (Van Halbeek et al., 1982). Presumably all these tissues should contain the core 3 β3-GlcNAc-transferase. However, of all the above tissues, only rat colon has been tested, and indeed, it is rich in the core 3 transferase. The data in Table VI suggest that pig, monkey, and human colonic mucins should contain the GlcNAcβ1–3GalNAc moiety.

When colon mucosal extracts from either rat or pig were tested for core 3 β3-GlcNAc-transferase activity by HPLC or NMR analyses, no GlcNAcβ1–6GalNAc-R product was detected. Further evidence suggesting that there is no enzyme which can add GlcNAc in β1–6 linkage to GalNAc-R (step c, Figure 6) is that the GlcNAcβ1–6GalNAc oligosaccharide has not as yet been isolated from a mucin except as a degradation product (Wu et al., 1984); this oligosaccharide always occurs as part of core 2 [Galβ1–3(GlcNAcβ1–6)GalNAc] or core 4 [GlcNAcβ1–3(GlcNAcβ1–6)GalNAc], indicating that core 1 is the precursor of core 2 and core 3 is the precursor of core 4 (Figure 6).

Incubation of rat colon mucosal extracts with GalNAc-α-benzyl or GalNAc-α-phenyl for 2 h (Brockhausen et al., 1983a) or with GalNAc-OSM for 3 h (the present study) produces two products, [$^{14}$C]GlcNAcβ1–3GalNAc-R and [$^{14}$C]GlcNAcβ1–3([$^{14}$C]GlcNAcβ1–6)GalNAc-R, indicating that rat colon contains both the core 3 β3-GlcNAc-transferase and core 4 β6-GlcNAc-transferase. Both enzymes can act on both low molecular weight acceptors (phenyl and benzyl glycosides) and mucins. It is difficult to carry out quantitative kinetics with the core 4 enzyme if its substrate must first be generated from GalNAc-R by the core 3 enzyme. Kinetic studies of the core 4 β6-GlcNAc-transferase were therefore carried out with GlcNAcβ1–3GalNAc-α-benzyl as acceptor.

Core 4 structures have been found in relatively few tissues, i.e., sheep gastric mucin (Hounsell et al., 1981) and human bronchial mucin (Van Halbeek et al., 1982), although the core 4 enzyme is widely distributed (Table VI). The relative scarcity of core 4 in mucins may be due to the limited distribution of the core 3 β3-GlcNAc-transferase (Table VI). The role of the core 4 enzyme in tissues with low levels of core 3 enzyme is probably mainly the synthesis of core 2 since it is highly likely that the core 2 and core 4 activities are due to a single enzyme.

There are, however, some discrepancies. For example, rat colon has both core 3 and core 4 transferase activities, and yet, rat colonic mucin contains core 3 but not core 4 structures (Slomiany et al., 1980). The core 3 structures in rat colonic mucin are sialylated, and it is therefore possible that sialyltransferase competes with core 4 β6-GlcNAc-transferase for common substrates. We attempted to verify this theory but could not detect transfer of sialic acid to GlcNAcβ1–3GalNAc-α-benzyl (path g, Figure 6). Since the sialylated structure is known to occur and since path f (Figure 6) has been ruled out, path g probably proceeds with mucin substrates but not with benzyl glycosides. The sialyltransferase that incorporates sialic acid in α2–6 linkage to the GalNAc residue of mucins (path e) can in fact act on GalNAc-R, Galβ1–3GalNAc-R, or sialylα2–3Galβ1–3GalNAc-R provided R is mucin polypeptide but is inactive with these substrates if R is a *p*-nitrophenyl group (Bergh & Van den Eijinden, 1983). The sialyltransferase activity we detected with Galβ1–3GalNAc-α-benzyl is therefore likely to be due to sialic acid incorporation in α2–3 linkage to the Gal residue.

Most GlcNAc-transferases require divalent cation for activity. The fact that added cation was not required for the core 4 GlcNAc-transferase may be due to the presence of tightly bound cation on the enzyme. The cation requirements of the core 2 and core 4 β6-GlcNAc-transferases are very similar (Table II), and competition experiments support the idea that the two activities are due to a single enzyme. It should be pointed out that the Galβ1–3GalNAc-α-benzyl substrate used to measure core 2 GlcNAc-transferase in these experiments is probably specific for this enzyme since it is not a substrate for the β3-GlcNAc-tranferase that adds GlcNAc in β1–3 linkage to Gal (Brockhausen et al., 1983b, 1984).

A tissue and species survey was carried out (Table VI) for the core 2 and core 4 GlcNAc-transferases. These data also indicate that the two activities are due to the same enzyme in a given species. The ratio of the two activities appears to be constant within a species but shows much variation between species. More tissues will have to be tested to verify this point. The high ratio of core 2 to core 4 activities in the dog submaxillary gland (Table VI) could be due to a factor that either activates the core 2 activity or inhibits the core 4 activity. Mixing experiments were carried out in which enzyme preparations from rat colon mucosa and dog submaxillary gland were assayed separately and together with either Galβ1–3GalNAc-benzyl or GlcNAcβ1–3GalNAc-benzyl as acceptors. The mixed incubations showed additive activities for both acceptors, indicating that canine submaxillary glands contained neither a stimulatory nor an inhibitory factor.

In summary, we have demonstrated for the first time the in vitro synthesis of mucin cores 3 and 4 and have defined some of the properties of the enzymes involved (Figure 6).

We present evidence in this paper that steps i and b (Figure 6) may be catalyzed by a single enzyme. No formation of GlcNAcβ1–6GalNAc-R was detected with GalNAc-α-R as a substrate when R was either benzyl or OSM polypeptide, and therefore, step c is indicated as a nonallowed reaction. Rat colon mucosal extracts transfer GlcNAc to GalNAc-α-benzyl (step a), GlcNAcβ1–3GalNAc-α-benzyl (step b), and GlcNAcβ1–6GalNAc-α-benzyl (step d) at rates of 7.9, 80.7, and 1.8 nmol h$^{-1}$ (mg of protein)$^{-1}$, respectively (Brockhausen et al., 1983a). Thus, path d occurs in vitro with benzyl glycoside at a relatively slow rate but probably has no physiological significance since GlcNAcβ1–6GalNAc-R is not synthesized. The physiological path for synthesis of core 4 is via paths a and b and of core 2 is via paths h and i. The synthesis of both core 2 and core 4 therefore requires substitution of carbon 3 of GalNAc prior to the substitution of carbon 6. It is interesting that substitution of carbon 6 of GalNAc by either GlcNAc (path c) or sialic acid (path e) greatly inhibits the action of the core 3 β3-GlcNAc-transferase (paths d and f, respectively). Path f can be ruled out because native OSM is not a GlcNAc acceptor under standard enzyme assay conditions. Similarly, it has previously been shown (Schachter & Williams, 1982) that substitution of carbon 6 of GalNAc by a sialic acid residue (step e) prevents Gal transfer to carbon 3 (step k) although sialylation after galactosylation (step j) occurs readily.

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# Tryptophan Imaging of Membrane Proteins†

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ABSTRACT: A theoretical analysis of resonance energy transfer between protein tryptophan and the *n*-(9-anthroyloxy) (AO) fatty acid probes has been carried out to evaluate its potential use in determining the tryptophan distribution in membrane proteins. The Förster theory for two-dimensional energy transfer was formulated to calculate multiple donor (tryptophan) transfer efficiencies to ensembles of AO probes at different depths in the bilayer. The variation of transfer efficiency with AO probe depth is found to be a sensitive function of tryptophan position and the protein radius but not the dipole–dipole orientation factor or the decay heterogeneity of the donor. For single tryptophan-containing proteins the model predicts that the tryptophan position can be determined with a precision of about 2 Å. Although for multiple tryptophans there is appreciable deterioration in resolution, it is still possible to determine the essential features of the distribution such as its first two moments. The positions determined by this method are the projections of the tryptophan positions on a plane perpendicular to the membrane surface, since the probes distribute uniformly around the protein. To analyze the data, a Monte Carlo approach has been developed to search for tryptophan distributions compatible with the observed efficiencies and to display the results in terms of a tryptophan density map. It is shown that even for cases in which little is known about the quantum yield distribution, significant information can be determined about the tryptophan spatial distribution.

Determination of the structure of membrane proteins is one of the most challenging problems in molecular biology. With few exceptions relatively little is known about the tertiary structure of proteins within the membrane. Tertiary structure information has been obtained for bacteriorhodopsin by combining the results of diffraction studies of the crystalline purple membrane, the primary structure, and theoretical arguments (Henderson & Unwin, 1975; Khorana et al., 1979; Ovchin-

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