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Purification and characterization of the MUC1 mucin-type glycoprotein, epitectin, from human urine: structures of the major oligosaccharide alditols

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The MUC1 glycoprotein, epitectin, a component of the human bladder epithelium, was purified from human urine. Sedimentation equilibrium analysis and gel filtration using polysaccharide or protein standards revealed a polydisperse preparation with molecular weights ranging from about 0.9 to 1.3×10^6. This suggests that in the native state epitectin exists as aggregates of three or four monomer units of 350–400 kDa. Epitectin was found to have significant affinity to hexyl-, octyl- or phenyl agarose indicating that hydrophobic interactions and possibly carbohydrate-carbohydrate interactions may be responsible for the self-association. Chemical and enzymic deglycosylation of [^{125}I]-labeled urine epitectin and metabolically labeled H.Ep.2 epitectin resulted in extremely polydisperse products. The buoyant densities of epitectin purified from urine and H.Ep.2 cells were found to be 1.39–1.40 g ml^{-1}, suggesting that the total carbohydrate content of these preparations is not significantly different. The O-linked saccharides of epitectin were fractionated by HPLC and analyzed by permethylation and FAB-MS. The neutral saccharides from both sources contain three common structures, namely Gal1 → 3GalNAc, GlcNAc1 → 6 (Gal1 → 3) GalNAc and Gal1 → 4 GlcNAc → 6 (Gal1 → 3)GalNAc. The sialic acid of urine epitectin consisted entirely of *N*-acetylneuraminic acid. The two sources of epitectin, *in vitro* labeled on sialic acid, were found to have the same sialyl oligosaccharides but in different proportions. Metabolic labeling and *N*-glycanase susceptibility experiments firmly established the presence of N-linked saccharides in epitectin as minor components. The remarkable similarities in the total carbohydrate content, the carbohydrate composition and structures of saccharides between epitectin from urine, a non-malignant source, and H.Ep.2 cells is surprising in view of the prevailing view that MUC1 glycoproteins of cancer cells are underglycosylated compared to those produced by non-malignant cells.

***Keywords*: MUC1 glycoprotein, mucins, human urine, carcinoma, oligosaccharide structures**

Introduction

The family of mucin-type sialoglycoproteins encoded by the *MUC1* gene was discovered by their interaction with monoclonal antibodies that distinguish between malignant and non-malignant cells [1–5]. Several investigators have cloned and sequenced the cDNA coding for the core protein of these glycoproteins in breast cancer cells [6, 7]. The cDNA codes for an extended central region of variable numbers of 20 amino acid tandem repeats with unique 3′ and 5′ sequences. It is believed that the differential interaction of the antibodies such as SM3 is primarily due to impaired glycosylation of these glycoproteins in the malignant cell [5]. This possibility is supported by the comparison of the saccharides of MUC1 glycoproteins isolated from BT-20 human breast carcinoma cells and human milk glycoprotein [8, 9]. It was found that the major saccharides of the breast cancer cells and human milk glycoproteins contained the type I (Galβ1 → 3GalNAc) core and type II (GlcNAcβ1 → 6 (Galβ1 → 3) GalNAc) core, respectively [8]. However, this is not an entirely satisfactory comparison because the milk glycoprotein may not be representative of the normal mammary epithelial cell surface, and also

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because the breast cancer glycoprotein was metabolically labeled using [^{3}H] glucosamine while the milk glycoprotein was *in vitro* labeled on galactose or sialic acid. To understand the mechanisms underlying the aberrant glycosylation of MUC1 glycoprotein in malignant cells, Brockhausen *et al.* [10] investigated the O-glycan chain synthesizing glycosyltransferases of an immortalized (normal) mammary epithelial cell line (MTSV1-7) and three human breast cancer cell lines. It was found that the CMP-sialic acid Galβ1 → 3GalNAc α-3-sialyltransferase was increased several fold in all three cancer cell lines compared to the control and two of the cancer cell lines had also lost the ability to synthesize the core-2 structures. Thus, it appears that at least in the case of human breast carcinoma, aberrant glycosylation of the MUC1 glycoprotein could be responsible for the unmasking of peptide epitopes.

We have previously purified epitectin, from metabolically labeled human laryngeal carcinoma (H.Ep.2) cells and elucidated the structures of its oligosaccharides [11, 12]. Our studies also demonstrated that epitectin is expressed on the luminal surface of human bladder epithelium and is shed into urine [11]. Subsequently, Swallow *et al.* [13] established that urinary epitectin is identical to the genetically polymorphic human urinary mucin-like glycoprotein, which was therefore re-named MUC1 glycoprotein/mucin. The extensive polymorphism of the MUC1 glycoprotein is believed to be due to variations in the number of tandem repeats and alternate splicing of the gene [6, 7]. MUC1 glycoprotein is also known to be expressed on other specialized normal epithelia such as the uterus, apocrine sweat glands, and the type II pneumocytes of the lung [14, 15]. It has been proposed that the MUC1 glycoproteins on normal epithelia and on malignant cells serve to protect cells from a potentially destructive physicochemical environment [11]. Other functions proposed for this cell surface glycoprotein include masking of target antigens from the immune system [16], masking of adhesion molecules and thus serving as anti-adhesive molecules [17] and regulation of cell growth [18]. Recently, we have demonstrated that the levels of epitectin in the urine of patients with interstitial cystitis, a bladder disorder of unknown etiology, are low compared to the levels in healthy controls [19]. Thus, even though human bladder/urine epitectin is a macromolecule of much interest it has not been fully characterized. Specifically, there is no information on either the structures of the carbohydrate chains of urine epitectin or the relationship of these structures to those found on epitectin from cancer cells.

In this contribution we report the purification of mg quantities of epitectin from pooled human urine and its biochemical characterization including structural investigation of the oligosaccharides released by β-elimination. The released oligosaccharide alditols were fractionated by HPLC and analyzed by permethylation and FAB-MS. Epitectin was also purified from large scale cultures of H.Ep.2 cells and its properties compared to that of the urinary material. We demonstrate that the epitectins from both sources are remarkably similar in their molecular size, carbohydrate content and saccharide structures.

Materials and methods

H.Ep.2 cells were obtained from the American Type Culture Collection (Gaithersburg, MD) [20]. Mouse monoclonal Ca2 antibody [3] was provided by Professor H. Harris, University of Oxford. HMFG-2 antibody [21] was purchased from AMAC, Inc. (Westbrook, ME). D-[6-^{3}H]-Glucosamine hydrochloride (26.8 Ci mmol^{-1}), [^{3}H] acetic anhydride (100 mCi mmol^{-1}), and D-[2,6-^{3}H] mannose (44 Ci mmol^{-1}) were from American Radiochemicals, Inc. (St Louis, MO). Eagle's minimum essential medium was from Flow Laboratories (McLean, VA). Fetal bovine serum, glutamine, sodium pyruvate, and non-essential amino acids were purchased from Gibco (Grand Island, NY). Amplify was purchased from Amersham (Arlington Heights, IL). Goat IgG and ELISA grade bovine serum albumin (BSA) were from Sigma Chemical (St. Louis, MO). Wheat germ agglutinin (WGA) and peanut agglutinin (PNA) conjugated to digoxigenin, anti-digoxigenin conjugated alkaline phosphase and goat anti-mouse IgG + IgM conjugated to peroxidase were from Boehringer Mannheim (Indianapolis, IN). Anti-mouse IgG (H + L)-Alkaline phosphatase was from Calbiochem (LaJolla, CA). PNA and PNA-Sepharose 4B (4 mg ml^{-1}) and ConA-Sepharose were from E-Y Laboratories (San Mateo, CA). The oligosaccharide standards, NeuNAc → Gal → (NeuNAc) → GalNAc(OH); NeuNAc → Gal → GalNAc(OH) and Gal → GalNAc(OH) were prepared from fetuin and glycophorin as described previously [22]. *N*-glycanase (peptide: *N*-glycosidase F) was purchased from Genzyme Corp. (Boston, MA) and endo-α-*N*-acetyl galactosaminidase was purified from culture filtrates of *Diplococcus pneumoniae* as described previously [23]. Phenyl-agarose and octyl-agarose were from Pharmacia LKB Biotech (Piscataway, NJ) and the dextran molecular weight standards were from Pharmacosmas (Viby Sj, Denmark).

Human urine

Pooled urine from healthy male individuals was collected in batches of 5–6 l in containers containing sodium azide (final concentration 0.02%) to prevent bacterial growth. The urine was stored overnight at 4 °C to cryoprecipitate, filtered through Whatman filter paper and concentrated to 100–200 ml by ultrafiltration at 4 °C using an Amicon XM-300 DiafloR Disc membrane (Amicon, Beverly, MA). When pure preparations of metabolically labeled epitectin were similarly subjected to ultrafiltration the recovery of the glycoprotein in the retentate was quantitative. The concentrate was dialyzed for 2 days at 4 °C with several changes of deionized water, lyophilized and the powder stored dry at

−20 °C until used for the isolation of urine epitectin. A total of 52 l of urine were processed in these studies.

Cell culture

H.Ep.2 cells were maintained as monolayers as described previously [20]. Metabolic labeling of the glycoproteins was accomplished by adding to the pre-confluent cells (48 h before harvest) medium (5 ml) containing [6-^{3}H]-glucosamine (10 μCi ml^{-1}) or [2, 6 ^{3}H] mannose (10 μCi ml^{-1}) and incubating as above. Large batches of H.Ep.2 cells were grown at the National Cell Culture Center, Cellex Biosciences, Inc. (Minneapolis, MN) on microcarrier beads in 8 l spinner culture vessels. The culture medium was the same as above except that 5% newborn bovine calf serum and 1% fetal bovine serum was substituted in place of the 10% fetal bovine serum, for economic reasons. Immunochemical measurements conducted on the trial batches of cells grown confirmed that there was no significant difference in the level of epitectin production under the above conditions of growth. The yield of cells was $3.2–4.3 \times 10^8$ cells per liter of culture and the viability of the cells was between 88 and 98%. A total about 7.4×10^9 cells were used for the isolation of H.Ep.2 epitectin.

Extraction of epitectin from H.Ep.2 cells

Cells were washed with PBS to remove serum components and suspended at 10^8 cells ml^{-1} of 50 mM Tris HCl, pH8.0 containing 0.1% Triton X-114, 1 mM phenylmethane sulfonyl fluoride, 1 mM benzamidine HCl, 2 mM *N*-ethylmaleimide and 5 mM EDTA and homogenized in a Dounce homogenizer. The homogenate was stirred at 4 °C for 1 h and centrifuged at 60 000 × **g** for 30 min. The supernatant was removed and the pellet extracted twice more as above. The combined supernatant was placed in a boiling water bath for 10 min to inactivate glycosidases and proteases [11, 12], the solution cooled and centrifuged again. The supernatant was used for epitectin isolation.

Purification of epitectin from urine concentrate and H.Ep.2 cell extracts

The first step in the isolation consisted of affinity chromatography on columns (1 × 5 cm) of PNA-Sepharose equilibrated with PBS-0.1% CHAPS. Separate columns were used for urine and cell extracts. The urine powder reconstituted in PBS-0.1% CHAPS or the above cell extract was applied on the column and recycled once. After incubation for 10 min, the unbound material was eluted with PBS-0.1% CHAPS until the eluate was free of absorbance of 280 nm. The column was then eluted with 4% galactose in PBS-0.1% CHAPS and the eluant collected in 1 ml fractions and aliquots analyzed for epitectin by ELISA using Ca2 mouse monoclonal antibody [3]. The positive fractions were pooled, dialyzed against water for 2 days and lyophilized to yield a crude preparation of epitectin.

Density gradient centrifugation

The centrifugation was carried out under dissociative conditions as previously described using CsTFA as the medium which, compared to CsBr, gave superior separation of mucins from proteins and nucleic acids [24].

Deglycosylation of epitectin

Chemical deglycosylation was carried out by the trifluoromethane sulfonic acid method as previously used by us for mucins [25, 26]. Enzymic deglycosylation was achieved by exhaustive sequential treatment with *V. cholerae* neuraminidase and *D. Pneumoniae* endo-α-*N*-acetyl galactosaminidase [23] in the presence of a mixture of protease inhibitors.

SDS-PAGE and electroblotting

The sample (10–100 μg) was treated with buffer containing 2% SDS and 0.1 M dithiothreitol (100 °C, 3 min) and subjected to electrophoresis on a 4–12% gradient slab gel at 30 mA for 5 h. The gels were stained with 0.05% Coomassie blue and destained. The glycoproteins were also detected by overlay with ^{125}I-lectins (WGA, PNA) followed by autoradiography. For the detection of [^{3}H]-labeled glycoproteins the gels were treated with Amplify, dehydrated and exposed to Kodak X-OMAT films. Electroblotting to nitrocellulose or polyvinylidene difluoride (PVDF) membrane was done in a Tris-glycine buffer, pH 8.9 (24 mM Trisbase, 192 mM glycine) containing 10% methanol at 120 mA, 18 h. The protein bands on the membrane were detected by staining with 0.1% amido black and the glycoproteins were visualized by incubation with lectin-digoxigenin or Ca2 MAb.

Column chromatography

BioGel A5m columns (1.4 × 86 cm) were equilibrated and eluted with 50 mM Tris HCl, pH 8.0 containing 0.01% SDS, 5 mM DTT and 4 mM EDTA. Sepharose CL-6B columns (1 × 50 cm) were equilibrated and eluted with 10 mM Tris HCl, pH 8.0, containing 0.1% sodium deoxycholate. FPLC was done on a Superose 6 column using 10 mM Tris-HCl pH 8.0 containing 0.1% CHAPS and the eluant screened at 280 nm. Oligosaccharides were fractionated on a BioGel P2 (200–400 mesh) column (1 × 110 cm) eluted with 0.1 M pyridine/0.1 M acetic acid, pH 5.0 and pre-calibrated with reference standards. In all cases, fractions were collected and aliquots analyzed for protein by the BCA assay, for radioactivity (radiolabeled samples) and for epitectin by ELISA as appropriate. Analysis of the fractions for dextran and neutral sugars was by the resorcinol-H_2SO_4 assay [27].

Hydrophobic interaction chromatography

The optimal conditions for the binding and elution of epitectin to phenyl-Sepharose and octyl-Sepharose were

determined by trial experiments based on recommendations by the suppliers (Pharmacia Biotech, Uppsala, Sweden). The sample was applied to the columns to 0.1 M potassium phosphate buffer, pH 5.0 containing 1.5 M ammonium sulfate. The bound glycoproteins were eluted with a gradient of ethylene glycol (see Figure 5 legend).

Iodination of urine epitectin

Small amounts (about 50 μg) of purified urine epitectin were radiolabeled by using Bolton-Hunter reagent [*N*-succinmidyl-3-(4-hydroxy, 5-[^{125}I] iodophenyl)propionate]. The ^{125}I-epitectin was repurified by gel filtration on Sepharose CL-6B and chromatography on DEAE-Sepharose. A portion of the iodinated epitectin was subjected to preparative SDS-PAGE and the individual 390 and 350 kDa forms of the glycoprotein recovered by electroelution of the appropriate sections of the gel.

In vitro labeling of sialyl residues on epitectin

The sialyl residues of human urine and H.Ep.2 epitectin (50 μg each) were modified to the 7- and 8-carbon analogs by mild treatment with sodium periodate (0.012 M, 4 °C, 10 min) and the generated aldehydes were reduced with NaB[^{3}H]$_4$ as described by van Lenten and Ashwell [28]. Chromatography of (^{3}H-sialyl)-labeled epitectin on a BioGel P-4 column before and after mild acid hydrolysis (0.05 N H_2SO_4, 80 °C, 1 h) demonstrated that at least 95% of the ^{3}H label was on modified sialic acid residues.

Alkaline-borohydride treatment of epitectin to release Ser/Thr-linked saccharides

Epitectin was treated with 1.0 M $NaBH_4$ in 0.05 M NaOH at 45 °C for 16 h or 37 °C for 72 h in an atmosphere of nitrogen. The reaction mixture was cooled in an ice bath, neutralized by dropwise addition of 4 M acetic acid, passed through a column of AG50 (H+) resin and the water eluate evaporated to dryness in a rotary evaporator. The residue was treated by repeated (three times) addition of methanol : HCI (1000 : 1) and evaporation to remove borate.

In vitro labeling of the oligosaccharide alditols derived from epitectin

The saccharides recovered as above were dissolved in 0.5 ml of water and passed through a SEP-PAK C18 cartridge (Millipore Corp.) to remove peptides. The eluted oligosaccharides were de-*N*-acetylated and then re-*N*-acetylated with [^{3}H] acetic anhydride as described by Amano *et al.* [29]. The radiolabeled saccharides were purified by spotting on a sheet of Whatman chromatography paper and eluting with butanol : ethanol : water (4 : 1 : 1) for 24 h. The saccharides which remained at the origin were recovered by elution with water and lyophilization.

High performance liquid chromatography (HPLC) separations of the [^{3}H]-acetyl labeled oligosaccharide-alditols

HPLC was performed on a quaternary amine-bonded silica (10 μM MicroPak AX-10) column (30×0.4 cm ID, Varian, Walnut Creek, CA). Radiolabeled samples were injected in 100 μl of water and eluted with 500 mM sodium phosphate as described by Baenziger and Natowicz [30]. Fractions of 1 ml were collected and 10 μl aliquots analyzed for radioactivity in a Beckman LS-3801 scintillation counter.

Further fractionation of the neutral and sialylated oligosaccharide-alditols was performed on primary amino-bonded silica (5 μm Amino Zorbax) column (4.6 mm ID×25 cm, Dupont Instruments, Wilmington, DE USA) with a Spectra-Physics model 8700 liquid chromatography apparatus (San Jose, CA) using different elutions for neutral and sialylated oligosaccharide-alditols. After equilibration with 70% of acetonitrile in water, neutral oligosaccharides were injected and eluted with the initial solvent for 10 min followed by a linear gradient to acetonitrile : water (60 : 40, by vol) for 60 min. The column was then eluted at isocratic conditions for 10 min and finally with a linear gradient to acetonitrile : water (50 : 50, by vol) for 10 min. Sialylated oligosaccharide-alditols were injected on a column equilibrated with 75% of acetonitrile in 15 mM KH_2PO_4, and eluted with the initial solvent for 25 min followed by a linear gradient to acetonitrile : 15 mM KH_2PO_4 (35 : 65, by vol) for 60 min. The flow rate was 1 ml per min. Fractions of one ml were collected and aliquots analyzed for radioactivity.

Amino acid and carbohydrate analysis

Amino acids were prepared for picotag analysis by vapor phase hydrolysis. The sample (0.5–10 μg) was dried in a 6×50 mm glass tube or a strip of PVDF containing the protein of interest placed in the tube. Several samples were placed in a vacuum vial containing 200 μl of 6 N HCl and a crystal of phenol. The vial was evacuated and flushed with nitrogen thrice before sealing under vacuum in the Waters Picotag work station. After hydrolysis at 112° for 24 h, the hydrolysates were dried and the amino acids derivatized to phenylthiocarbamyl amino acids. The PTC-amino acids were analyzed on a Waters picotag column at 39 °C and a Hewlett Packard HP1090 system with a diode array detector. The elution system described by Gupta and Jentoff [31] was used for effective separation and quantitation of the amino acids and hexosamines.

Carbohydrate analysis was done by high-performance-anion-exchange chromatography (HPAEC) using a Dionex System equipped with a pulse amperometric detector, as described by Lee [32]. For neutral sugars and amino sugars, samples were hydrolyzed in 2 M trifluoroacetic acid at 100 °C for 8 h. The dried hydrolysates were dissolved in water and analyzed on a Carbo Pac PA-1 column by isocratic elution with 16 mM sodium hydroxide. Sialic acids

(NeuNAc and NeuNGly) were determined after hydrolysis in 0.1 N H_2SO_4 at 80 °C for 1 h. The hydrolysate was neutralized with NaOH and an aliquot analyzed by HPAEC.

Methylation analysis

The oligosaccharide-alditols purified on HPLC were methylated according to Ciucanu and Kerek [33] with methyl iodide, solid NaOH, and methylsulfoxide. The partially methylated and acetylated methyl glycosides were identified by gas-liquid chromatography/mass spectrometry (GLC/MS) according to Fournet *et al.* [34].

Fast atom bombardment mass spectrometry

A Concept II H-H mass spectrometer (Kratos Analytical Instrument, Urmstom, Manchester, UK) equipped with a DS 90 (DGDG/30) data system was used in these studies. The mass spectrometer was operated by a 8 kV accelerating potential. An ion tech model B11 NF saddle field fast atom source energized with the B 50 current-regulated power supply was used with xenon employed as the bombarding atom (operating condition, 7.3 kV, 1.2 mA). The mass range 2000–200 was scanned at 10 s per decade. The methylated oligosaccharide-alditols were analyzed in positive mode using thioglycerol 0.5 M NaI as matrix.

Results and discussion

Purification of epitectin

After affinity chromatography on PNA-Sepharose of urine concentrated by ultrafiltration through 300 kDa cut-off membrane, one liter of urine yielded an average of 250 μg material which eluted with 4% galactose. Examination of this material by polyacrylamide electrophoresis and gel filtration revealed the presence of low molecular weight (<200 000) proteins and glycoproteins in addition to epitectin. The samples purified by antibody affinity chromatography [11, 12] showed a similar pattern on SDS-PAGE as those purified by lectin affinity. Repeated affinity chromatography using various buffers including those containing a high concentration of salts, detergents etc, did not remove the low molecular glycoproteins completely. Fast protein liquid chromatography on a Superose 6 (HR 10/30) column (Figure 1) or gel filtration on Sepharose CL-6B in buffer containing 0.1% CHAPS was employed to separate epitectin from the low molecular weight material. In the final purification step the high molecular weight material was dissolved in 50 mM Tris HCl, pH 8.0 and applied on a column of DEAE-Sepharose. After eluting small amounts of the unbound material (which did not interact with the (Ca2 antibody) with the above buffer, epitectin was recovered by elution with 0.2 M NaCl. Gel electrophoresis on 4–12% gradient gels followed by staining of the gel with Coomassie blue, silver nitrate or of electroblots with amido black confirmed the complete removal of low molecular weight

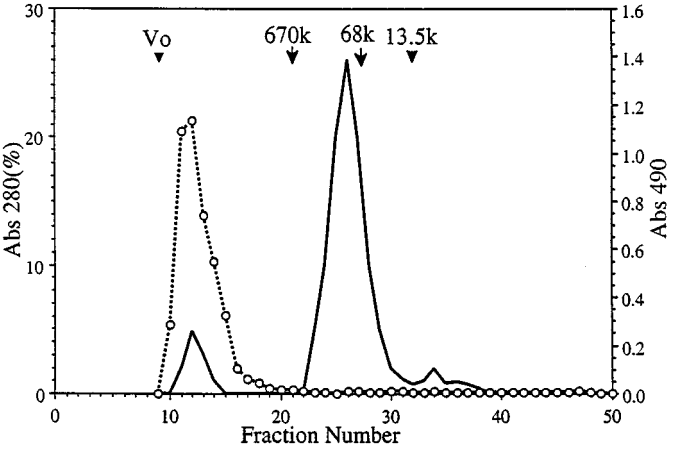


Figure 1. FPLC of a crude preparation of urine epitectin. The material isolated by affinity chromatography of urine concentrate on a column of PNA-Sepharose was applied on a column of Superose 6 (HR 10/30) and eluted with 50 mM Tris HCl, pH 8.0 containing 0.1% CHAPS. The eluate was scanned for absorption at 280 nm (——), collected in 1 ml fractions and aliquots analyzed for epitectin by ELISA (Abs 490) using Ca2 antibody (- - -○- -○- - -). The peak elution positions of protein molecular weight standards are indicated by arrows.

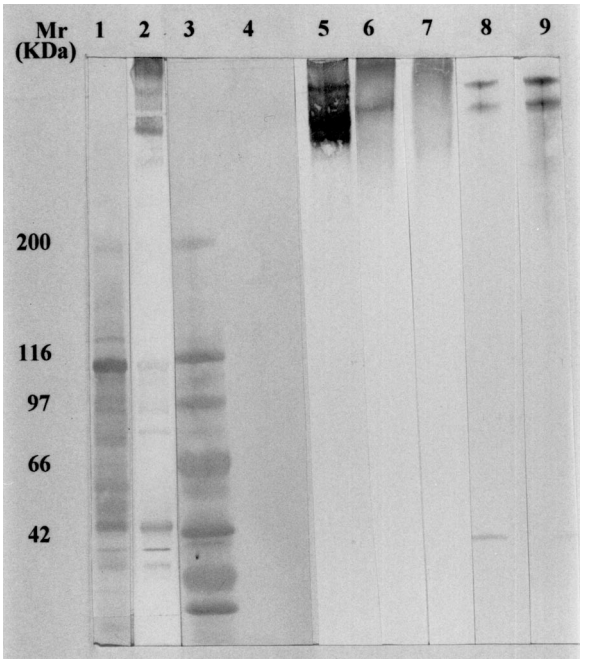


Figure 2. SDS-PAGE of urine and H.Ep.2 cell epitectin. The samples were subjected to electrophoresis on 4–12% gradient slab gels and transferred to nitrocellulose. Urine concentrate (lane 1 and 2); protein standards (lane 3); purified urine epitectin (lanes 4–7); purified H.Ep.2 cell epitectin (lanes 8 and 9). The proteins and glycoproteins were visualized by staining with Amido black (lanes 1, 3 and 4), overlay with Ca2 antibody (lanes 2, 7 and 9), PNA-digoxigenin (lanes 6, 8) or WGA-digoxigenin (lane 5).

protein/glycoprotein contaminants (Figure 2). Treatment of the electroblots with Ca2 antibody, PNA-digoxigenin or WGA-digoxigenin revealed the multiple diffuse bands of epitectin in the 350 000 to 400 000 molecular mass range

(Figure 2). The final yield of purified urinary epitectin was between 20 and 50 $\mu g\,l^{-1}$. Epitectin from H.Ep.2 cells was purified in a similar manner yielding 980 μg of epitectin per 10^9 cells which was also found to be free of contaminants when tested as above (Figure 2).

Low molecular weight glycoprotein from H.Ep.2 which co-purifies with epitectin

The nature of the low molecular weight glycoproteins was further investigated by growing H.Ep.2 cells in the presence of [^{3}H] $GlcNH_2$ and isolating these components by affinity chromatography followed by gel filtration of the cell extracts. A major low molecular weight glycoprotein of about 115 000 was partially purified. It had a [^{3}H] $GalNH_2$: [^{3}H] $GlcNH_2$ ratio of 1 : 3.7 in contrast to about 9 : 1 for the purified H.Ep.2 epitectin. When H.Ep.2 cells were grown in the presence of [^{3}H] mannose, the ^{3}H activity in the low molecular weight component was about 15-fold higher compared to that in epitectin. The high mannose content of the low molecular weight glycoprotein was also indicated by its strong affinity to ConA, whereas epitectin did not interact with this lectin. Further, in contrast to epitectin, the major portion of the saccharides in the low molecular weight glycoproteins are susceptible to *N*-glycanase consistent with the higher mannose and glucosamine content. These results confirm that the low molecular weight glycoproteins are not degradation products of epitectin but are glycoproteins with distinct amino acid and carbohydrate composition. The co-elution of these glycoproteins and epitectin suggests that they may be present as a complex with strong affinity to each other. Other investigators have demonstrated the existence of mucin glycoproteins such as ASGP-I and HMFG at the cell surface as complexes with low molecular weight glycoproteins [35, 36].

Molecular weight of epitectin

The molecular weights in the range of 350 000 to 400 000 previously assigned to epitectin and other MUC1 glycoproteins are based on their mobility in SDS-PAGE and therefore do not indicate the true value for two reasons. One, heavily glycosylated glycoproteins behave anomalously in SDS-PAGE [37] and two, the values are based on non-glycosylated protein standards. When examined by equilibrium sedimentation analysis [38] the molecular weights of urine and H.Ep.2 cell epitectin were found to be 1.3×10^6 and 1.5×10^6, respectively. The plots of the log of concentration versus (radius)^2 are non-linear, indicating some degree of polydispersity of the epitectin samples (Figure 3). The molecular weight of urine epitectin based on its elution from a Superose 6 column in FPLC and in comparison to protein standards was calculated to be $\sim1\times10^6$ (Figure 1). The elution of urine epitectin on a BioGel A5m column under denaturing and reducing conditions is illustrated in Figure 4. Based on the elution of the dextran (polysaccharide) standard, the molecular weights of the two partially separated species were estimated to be 1.25×10^6 and 0.9×10^6, respectively. The results of SDS-PAGE and overlay of the gel with ^{125}I-WGA of portions of the pooled fractions are also shown in Figure 4. It can be seen that gel filtration under SDS denaturing conditions resulted in the release of small amounts of material containing a series of components with molecular weight in the range of 90 000–40 000. It will be interesting to determine whether these species are generated as the result of breakage of specific peptide bonds in the tandem repeat segments of the MUC1 core protein [5, 6].

Hydrophobic interaction chromatography

The three- to four-fold higher molecular weight of epitectin obtained by sedimentation equilibrium or gel filtration under different conditions compared to values obtained by SDS-PAGE suggested self-association of the epitectin molecules. In order to obtain information on the nature of the interactions causing the self association, the behavior of urine epitectin (*in vitro* labeled with ^{125}I) in hydrophobic interaction chromatography was examined [39]. The results

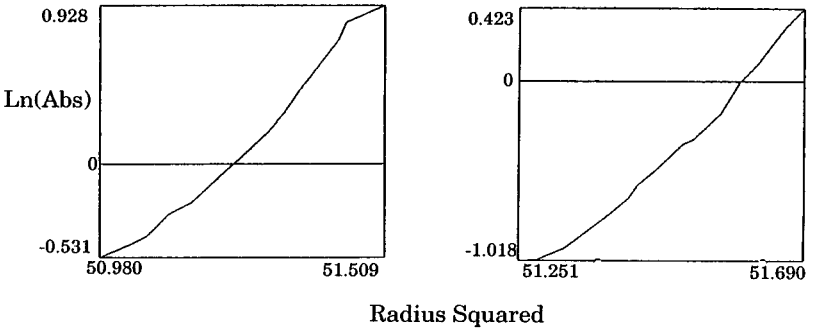


Figure 3. Equilibrium sedimentation analysis of epitectin. The urine (left panel) and H.Ep.2 (right panel) epitectins were analyzed in a double-sector cell using interference optics [38]. The samples were dissolved and equilibrated in 50 mM Tris HCl, 10 mM EDTA, 200 mM KCl with 0.1% CHAPS. The centrifugation was performed in a Beckman Optima XLA analytical ultracentrifuge at 5000 rpm, 21 °C for 72 h. The material was recovered after centrifugation by dialysis and lyophilization.

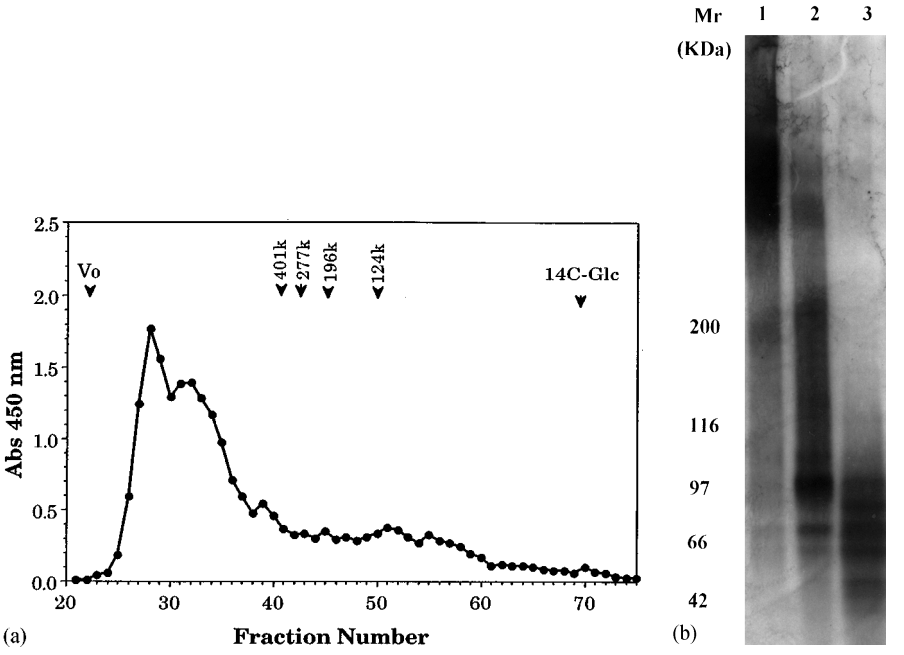


Figure 4. Chromatography of purified urine epitectin on a column of Bio Gel A5m under reducing and denaturing conditions. The sample was dissolved in 1 ml of 50 mM Tris HCl, pH 8.0 containing 2% SDS, 0.1 M DTT, and heated at 100 °C for 5 min and centrifuged. The supernatant was applied on the column and eluted with 50 mM Tris HCl, pH 8.0 containing 0.01% SDS, 5 mM DTT and 4 mM EDTA. Fractions of 2 ml were collected and aliquots analyzed for epitectin by ELISA (Abs 450 nM) using Ca2 antibody ●——●). The peak elution positions of dextran molecular weight standards chromatographed under identical conditions but detected by a resorcinol-H_2SO_4 assay [27] are indicated by arrows (left panel). The material in fractions 25–36, 37–47 and 48–60 were recovered and portions subjected to SDS-PAGE on a 4–12% gradient gel. The glycoproteins were visualized by overlay with ^{125}I-WGA, lane 1 (fr. 25–36); lane 2 (fr. 37–47) and lane 3 (fr. 48–60) (right panel).

of representative experiments on the interaction of urine epitectin with octyl- and phenyl-agarose illustrated in Figure 5 suggest the presence of hydrophobic sites in epitectin. However, not all molecules of epitectin have hydrophobic sites since on rechromatography the recovered unbound material still did not bind. It is possible that differences in the core protein such as the presence or the absence of the transmembrane segment [7, 15], as well as variations in the types and distribution of the saccharides, are responsible for the presence of hydrophobic sites on some but not all epitectin molecules. The presence of hydrophobic sites in epithelial mucins such as ovine submaxillary, canine tracheal and human respiratory mucins has been documented [40, 41].

CsTFA density gradient centrifugation

The purified ^{125}I-labeled urine epitectin when subjected to density gradient centrifugation revealed the presence of heterogeneous species with an average buoyant density of 1.39 g ml^{-1} (Figure 6). The ^{125}I-labeled epitectin after deglycosylation by treatment with trifluoromethane sulfonic acid was found to sediment at the top of the tube with density ($\leqslant$1.3 g ml^{-1}) similar to that of bovine serum albumin. In previous studies the density of metabolically

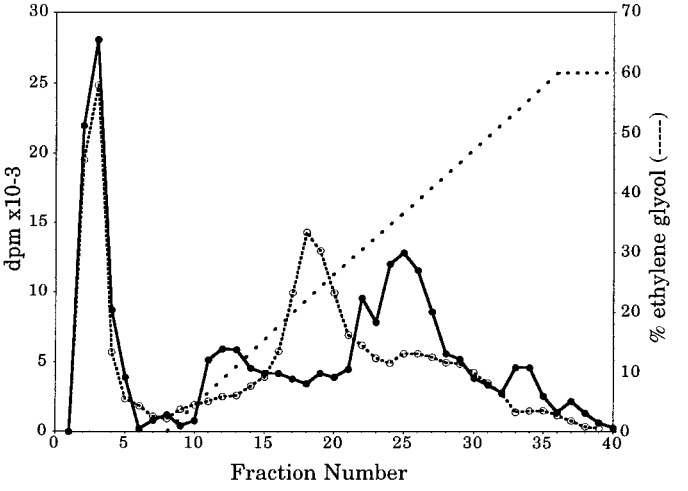


Figure 5. Hydrophobic interaction chromatography of purified ^{125}I-labeled urine epitectin. The sample was applied to a column (1.0×1.3 cm) of phenyl-Sepharose (○----○) or octyl Sepharose (●–––●), equilibrated with 0.1 M potassium phosphate buffer pH 5.0 containing 1.5 M ammonium sulfate. The column was eluted with the above buffer (8 ml), followed by a gradient (·····) of the same buffer (15 ml) to 60% aqueous ethylene glycol (15 ml) and finally with 60% aqueous ethylene glycol. Fractions of 1 ml were collected and analyzed for radioactivity in a gamma counter.

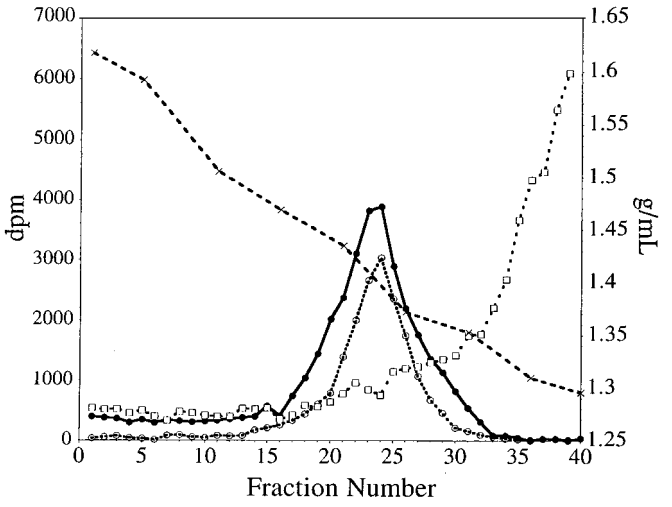


Figure 6. Density gradient centrifugation of urine epitectin. The purified ^{125}I-labeled urine epitectin or the deglycosylated material was dissolved in 16.7 mM phosphate buffer, pH 6.8 containing 4 M guanidinium chloride, 33 mM NaCl, 0.02% NaN_3, and 47% (w/w) CsTFA. The mixture was centrifuged at 14 °C in a Beckman 60Ti rotor for 72 h at 42 0000 rpm (~130 000 × **g** average). Fractions of about 0.8 ml were collected by gentle aspiration from the bottom of the tube and densities (×----×) of every fifth fraction were determined by weighing 100 µl in a calibrated micropipet. The fractions were analyzed for ^{125}I radioactivity in a Gamma counter. (●–––●, native urine epitectin; □·····□, urine epitectin deglycosylated by treatment with TFMS). The result of the centrifugation of ^{3}H-labeled H.Ep.2 cell epitectin under identical conditions is superimposed for comparison (○----○).

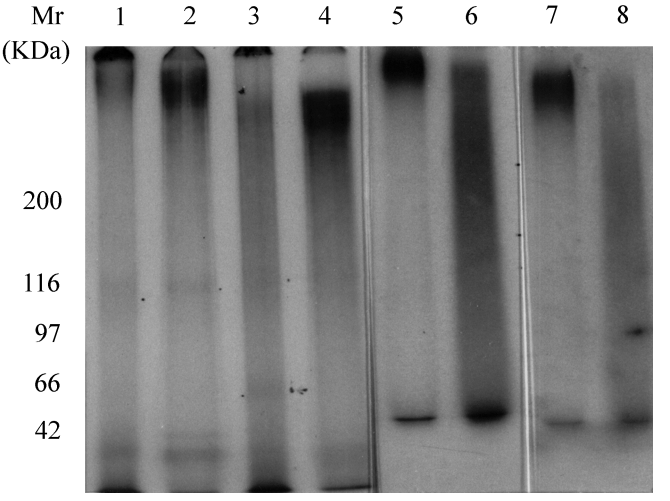


Figure 7. Deglycosylation of ^{125}I-labeled urine epitectin. SDS-PAGE of ^{125}I-labeled 390 kDa and 350 kDa species of urine epitectin before and after chemical (TFMS, 3 h, 25 °C) or enzymic (neuraminidase and endo-α-*N*-acetylgalactosaminidase) deglycosylation. The samples were subjected to electrophoresis on a 4–12% gradient gel followed by autoradiography. Lanes 2 and 5, 390 K epitectin before treatment; lane 1, 390 K epitectin after treatment with enzymes; lane 6, 390 K epitectin after treatment with TFMS; lane 4 and 7, 350 K epitectin before treatment; lane 3, 350 K epitectin after treatment with enzymes and lane 8, 350 K epitectin after treatment with TFMS.

labeled epitectin from H.Ep.2 was found to be 1.39–1.4 g ml^{-1} in a CsBr medium [12]. We reanalyzed the H.Ep.2 epitectin by CsTFA density gradient centrifugation for direct comparison with urine epitectin and found that the peaks are coincident indicating identical buoyant density on CsTFA for both preparations (Figure 6). This suggests very similar carbohydrate content, probably of the order of 50%, for both preparations.

Core protein(s) of urine epitectin

SDS-PAGE of the chemically deglycosylated ^{125}I-labeled epitectin on a 4–12% gradient gel revealed streaks extending from the top to the bottom of the gel as illustrated for the 390 kDa and 350 kDa epitectin bands (Figure 7). We have previously established that the chemical conditions used (TFMS, 25 °C, 3h) result in 90–95% deglycosylation of mucins without detectable polypeptide degradation [25, 26]. SDS-PAGE of ^{125}I-epitectin samples subjected to enzymic deglycosylation also showed no distinct band of the deglycosylated epitectin, instead the disappearance of the native glycoprotein was accompanied by appearance of faint streaks, and a small amount of radioactive material remaining at the top of the gel which may be aggregates (Figure 7). When epitectin isolated from H.Ep.2 cells metabolically labeled with [^{3}H] proline was treated with neuraminidase and endo-α-*N*-acetylgalactosaminidase, once again the deglycosylated products showed long faint streaks (not illustrated). When [^{3}H]-epitectin purified from H.Ep.2 cells metabolically labeled with [^{3}H] $GlcNH_2$ was treated with the above enzymes, greater than 90% of the radioactivity was released as saccharides in agreement with our previously reported results [12]. In general, deglycosylation studies on mucin glycoprotein yield results which cannot be readily explained. When epithelial mucins with molecular weights of several millions are deglycosylated, the products are either very low molecular weights [25, 26, 42] or else are highly polydisperse products [43–45].

Amino acid and monosaccharide compositions

Table 1 summarizes the amino acid and carbohydrate composition of the two epitectins determined by picotag and HPAEC analysis, respectively. The carbohydrate compositions of the two preparations are not very different as indicated by the molar ratios of the monosaccharide components except for the slightly higher GlcNAc and Galactose content of urine epitectin compared to the cancer cell epitectin (Table 1). HPAEC analysis of the sialic acid released from urine epitectin by mild acid hydrolysis revealed a single peak with mobility identical to that of *N*-acetylneuraminic acid [46]. Previously, we had established that the sialic acid released by neuraminidase from metabolically labeled H.Ep.2 epitectin is composed entirely of

Table 1. Compositional analysis of urine and H.Ep.2 cell epitectin

	Urine	*H.Ep.2 cell*
	(Residues/100 residues)[a]	
Aspartic/asparagine	5.2	6.1
Threonine	10.8	13.3
Serine	13.2	13.5
Glutamic/glutamine	4.0	2.1
Proline	13.4	12.1
Glycine	14.1	15.2
Alanine	14.2	13.9
Valine	6.0	5.5
Isoleucine	0.5	1.8
Leucine	3.3	3.2
Tyrosine	1.3	0.6
Phenylalanine	2.6	1.8
Histidine	3.9	3.5
Lysine	2.8	2.6
Arginine	4.7	4.8
	Urine	*H.Ep.2 cell*
	(Molar ratio)[b]	
Sialic acid	0.90	1.07
GalNAc	1.00	1.00
GlcNAc	0.54	0.24
Galactose	1.50	1.16
Mannose	0.29	0.18
Fucose	0.08	0.05

[a]Average of three separate analyses for urine epitectin and two analyses for H.Ep.2 cell epitectin. Trace amounts of cysteic acid and methionine were present but could not be quantitated.
[b]Estimated by HPAEC analysis of purified epitectin as described in the text. Molar ratio with reference to GalNAc as 1.00, average of three analyses.

N-acetylneuraminic acid [12]. The amino acid composition of the two epitectin preparations is compatible with that of other mucin-type glycoproteins, with Ser, Thr, Pro, Ala and Gly comprising 65.7 and 68% of the total (Table 1). This composition is consistent with the deduced amino acid sequence of MUC-1 glycoprotein containing 40 tandem repeats, in which the above five amino acids comprise about 70% of the total [4]. Considering that epitectin from pooled urine is a mixture of various polymorphic forms with unknown numbers of tandem repeats, the agreement between the above values are very good. When the amino acid composition of urine epitectin was determined after treatment with mild alkali to β-eliminate the saccharides, the values for serine and threonine were decreased by 16 and 54%, respectively. The higher proportion of threonine destroyed is interesting in view of the findings that UDP-GalNAc: polypeptide GalNAc transferase preferentially glycosylates threonine compared to serine in synthetic and natural peptides [47–49].

Presence of Asn-linked saccharides in epitectin

The cDNA sequence of MUC1 glycoprotein reveals five potential *N*-glycosylation sites [6, 7, 17]. However, whether any of these sites are indeed glycosylated has not been established unequivocally. Our highly purified epitectin preparations revealed the presence of mannose (Table 1), strongly suggesting the presence of some Asn-linked saccharides. To confirm that mannose is a component of epitectin and not of trace contaminant glycoproteins, extracts of H.Ep.2 cells grown in the presence of [^{3}H] mannose were immunoprecipitated with MUC1-specific antibodies and subjected to SDS-PAGE. Autoradiography revealed two bands with molecular weights of 390 000 and 350 000 characteristic of epitectin (Figure 8). The nature of the incorporated radiolabel was investigated by acid hydrolysis (3 M HCl, 100 °C, 8 h) of the immunoprecipitated epitectin and paper chromatography (Figure 8). The major portion of the radiolabeled product migrated in the position of reference mannose with some labeled material migrating in the position of fucose, a known metabolite of mannose. The slower moving (15–21 cm) labeled material is probably mannose-containing oligosaccharides librated by acid hydrolysis. In other experiments, epitectin purified from extracts of H.Ep.2 cells grown in the presence of [^{3}H] $GlcNH_2$ was treated with *N*-glycanase (as per instructions of the supplier) and the mixture chromatographed on a column of Sephacryl S-200 (data not illustrated). About four percent of radioactivity was found in the oligosaccharides released by the enzyme and eluting in the included volume of the Sephacryl S-200 column. There was no release of radioactive material in a control in which the epitectin was incubated with the buffer only. Since both the 390 and 350 kDa forms of epitectin are labeled with mannose, *N*-glycosylation is not confined to a specific glycoform of epitectin. The presence of some N-linked saccharides even in very heavily *O*-glycosylated mucin glycoprotein has led to speculations on possible function. In a preliminary experiment we examined the effect of inhibition of *N*-glycosylation on the expression of epitectin by H.Ep.2 cells. H.Ep.2 cells were cultured either in the absence or presence of tunicamycin. The cells were harvested and levels of cell surface epitectin examined by direct immunofluorescent labeling and FACS analysis. The tunicamycin-treated cells had slightly enhanced Ca2 Ab binding suggesting that *N*-glycosylation is not essential for the transport of epitectin to the cell surface (results not illustrated). The slight increase in Ca2 Ab binding to tunicamycin-treated cells could be due to unmasking of epitopes that were masked by other *N*-glycosylated cell surface glycoproteins. These results are in contrast to those of Hilkens and Buijs [50] suggesting a role for *N*-glycosylation in the biosynthesis of MUC1 glycoprotein by mammary tumor cells.

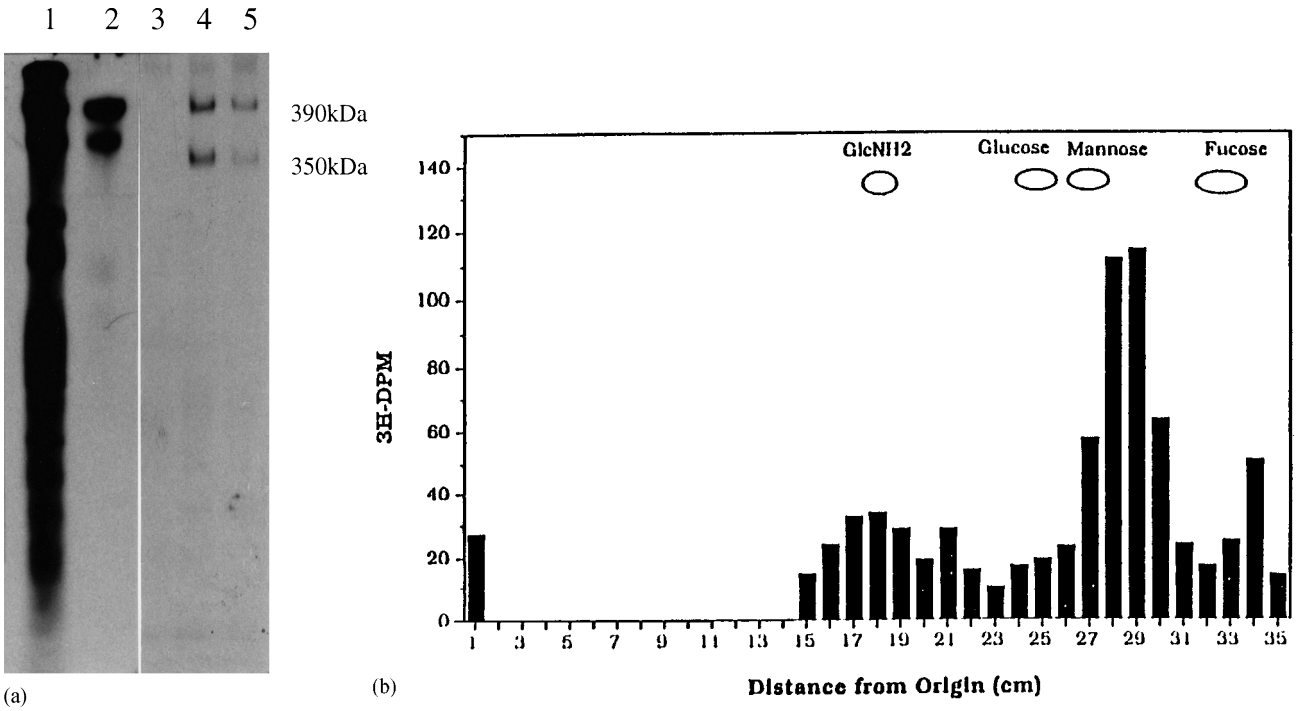


Figure 8. SDS-PAGE of immunoprecipitated metabolically labeled epitectin. Extracts of H.Ep.2 cells cultured in the presence of [^{3}H] mannose (lanes 3, 4, 5) or [^{3}H] glucosamine (lane 2) were immunoprecipitated with Ca2 antibody (lanes 2 and 4), HMFG-2 antibody (lane 5) or nonspecific mouse immunoglobulin (lane 3) and Protein A-agarose prearmed with rabbit antimouse immunoglobulin. The pellet was collected, washed three times with 50 mM Tris HCl, pH 8.0 containing 0.1 M NaCl, 1 mM EDTA, 0.5% NP-40 and 1 mM PMSF and treated with SDS-sample buffer at 100 °C for 3 min. The mixtures were centrifuged and the supernatants and total cell extract (lane 1) subjected to electrophoresis on a 4–12% gradient gel followed by fluorography (left panel). The epitectin immunoprecipitated from [^{3}H] mannose labeled H.Ep.2 cells was hydrolysed (3 N HCl, 100 °C, 8 h) and the dried hydrolysate subjected to paper chromatography using pyridine: ethylacetate: water: glacial acetic acid (5 : 5 : 3 : 1) as the solvent. To detect radioactive samples, the chromatogram was cut into 1 cm strips, extracted with 1 ml of water and analyzed by liquid scintillation counting. Reference sugars were located by spraying with an alkaline-silver nitrate solution (right panel).

Comparison of oligosaccharide alditols prepared by mild alkaline-borohydride treatment of [^{3}H-sialyl] labeled urine and H.Ep.2 epitectin

The products of the mild alkaline borohydride treatment were mixed with [^{14}C] glucose and examined by gel filtration on a column of BioGel P-6 (Figure 9). The elution profiles were very similar but the distribution of the radioactivity in the different peaks, however, varied for the two preparations. The saccharide alditols from urine epitectin were fractionated into neutral, monosialylated and disialylated species by chromatography on QAE-Sephadex. The monosialylated and disialylated saccharides were further fractioned on columns of BioGel P4 (minus 400 mesh) and the components tentatively identified on the basis of their elution positions compared to saccharide alditols of known structures isolated from glycophorin, fetuin, ovine submaxillary mucin and asialofetuin [22, 51]. The results are summarized in Table 2.

Fractionation of unlabeled oligosaccharide alditols

In order to monitor the purification and fractionation of the very small amount of individual saccharide alditols released from unlabeled epitectin, they were radiolabeled *in vitro* with [^{3}H] acetic anhydride [29]. This approach will label all saccharides since each saccharide should contain minimally one *N*-acetylated sugar in the form of *N*-acetyl galactosaminitol. Alkaline borohydride degradation of 1.6 mg urine epitectin followed by *in vitro* [^{3}H]-labeling with [^{3}H] acetic anhydride yielded 900 µg of the pure oligosaccharide alditols. Similarly the [^{3}H]-labeled oligosaccharide alditols were also obtained from 0.98 mg of H.Ep.2 cell epitectin. The mixture of oligosaccharide alditols in each case was separated into neutral (FN) and acidic (FA) fractions by HPLC on an AX-10 column. These fractions were then subjected to HPLC on an Amino-Zorbax column yielding four fractions each as illustrated for the urine epitectin saccharides (Fig. 10).

Structural determination of the major oligosaccharide-alditols

Fractions FN-1, FN-3 and FN-4 were methylated and a portion analyzed by FAB-MS in the positive mode using thioglycerol in the presence of 1% trifluoroacetate as matrix to identify the pseudo-molecular ions. The balance of the

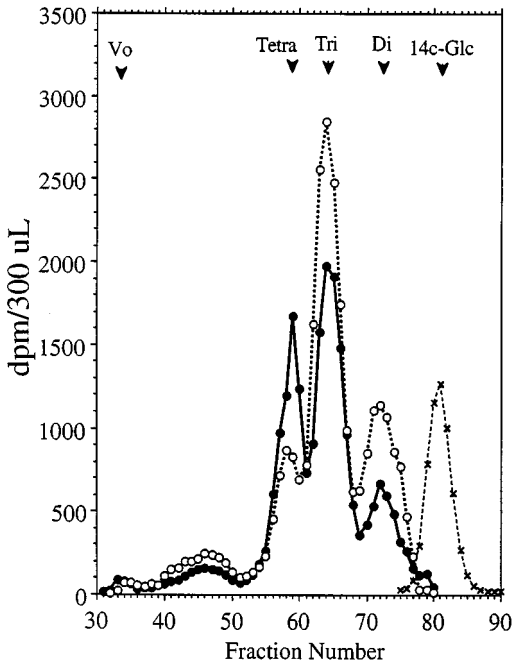


Figure 9. Gel filtration of saccharide alditols from urine and H.Ep.2 cell epitectin. Epitectin ^{3}H-labeled *in vitro* on sialyl residues was subjected to mild alkaline borohydride treatment as described in the text. The products were mixed with [^{14}C] glucose and subjected to chromatography on a column (0.9×110 cm) of Bio Gel P-6 and eluted with 0.1 N pyridine/acetic acid. Fractions were collected and aliquots analyzed for radioactivity. The superimposed elution profiles of urine epitectin (○····○) and H.Ep.2 cell epitectin (●——●) using the elution position of ^{14}C-glucose as reference are illustrated.

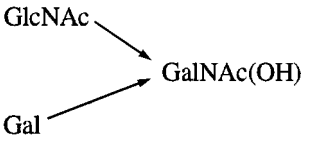
methylated oligosaccharide was subjected to methanolysis and the partially methylated and acetylated methyl glycosides were identified by gas liquid chromatography/mass spectrometry.

Fraction FN-1

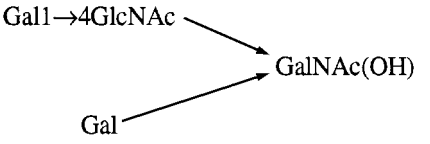
The FAB-MS showed the presence of a pseudomolecular ion at *m/z* 534 which is probably composed of one each of methylated hexose and *N*-acetyl hexosaminitol and Na^+. The methylated saccharides obtained after hydrolysis were 2,3,4,6-tetra-*O*-methyl galactose and 1,4,5,6-tetra-*O*-methyl *N*-acetyl galactosaminitol. These results establish the structure of this neutral oligosaccharide as Gal1 → 3 GalNAc(OH).

Fraction FN-3

This fraction gave two pseudomolecular ions at *m/z* 779 and 983. These ions can be accounted for by two oligosaccharide-alditols consisting of Hex(1), HexNAc(1) HexNAc-ol(1) and Na and Hex(2), HexNAc(1), HexNAc-ol(1) and Na, respectively. The partially methylated saccharides produced by hydrolysis were identified as 2,3,4,6-tetra-*O*-methyl galactose, 3,6-di-*O*-methyl *N*-acetyl glucosamine and 1,4,5-tri-*O*-methyl-*N*-acetyl galactosaminitol. Based on these results the sequences of the oligosaccharides present in this fraction are deduced as



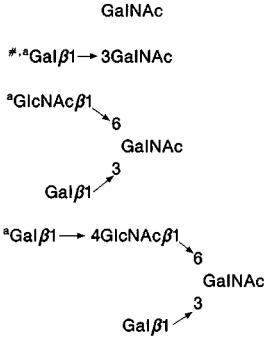
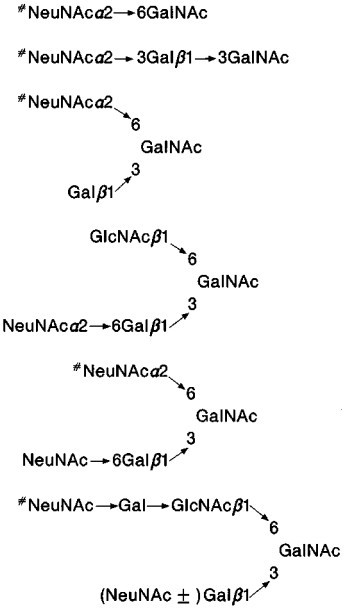
and



Fraction FN-4

This fraction gave a major ion at *m/z* 983 and the partially methylated, acetylated saccharides obtained by GC/MS analysis suggests that this fraction is the same tetrasaccharide found in FN-3.

Table 2. Neutral and acidic saccharides of urine and H.Ep.2 cell epitectin

Neutral	*Acidic*[b]
	

[a]These structures were determined by permethylation and FAB-MS analysis.
[b]The acidic saccharides were only tentatively identified by their gel filtration elution positions in relation to calibration standards. However, the ones marked # have been previously purified from metabolically labeled H.Ep.2 cell epitectin and further characterized [12].

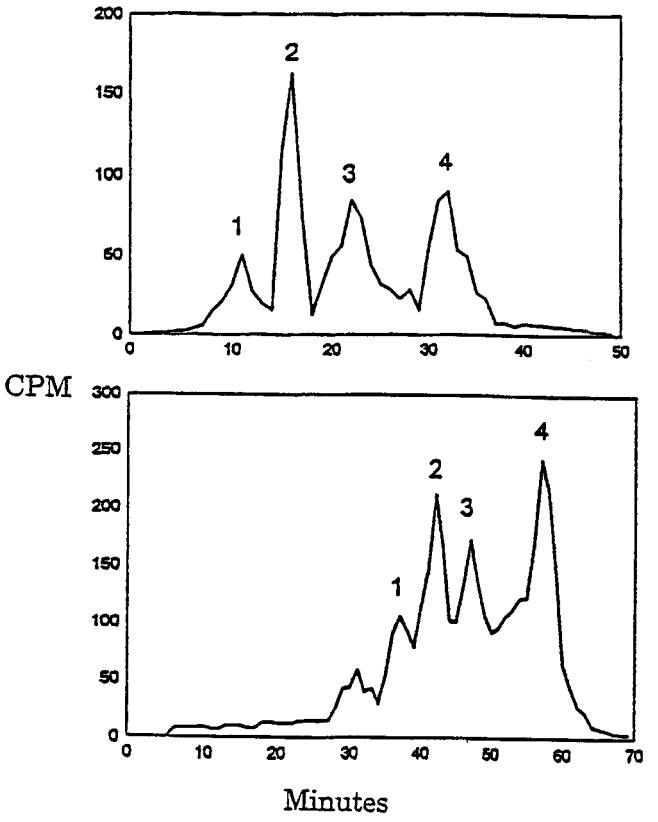


Figure 10. HPLC fractionation of the ^{3}H-labeled oligosaccharide alditols. The ^{3}H-labeled saccharide samples were prepared from urine epitectin as described in the text and separated into the neutral and acidic fractions by HPLC on a column of AX-10. The neutral (upper panel) and acidic (lower panel) fractions were further fractionated into four fractions each (FN-1, FN-2 *etc.*) by HPLC on an Amino-Zorbax column as described in the text.

Acidic oligosaccharide-alditol

Of the four fractions (FA-1 to FA-4) from urine epitectin only fraction FA-1 was present in sufficient quantity to be examined by FAB/MS. It showed a pseudomolecular ion at m/z 1140 which is consistent with a tetrasaccharide composed of NeuNAc(1), Hex(1), HexNAc(1) and HexNAcol(1). The partially methylated, acetylated methyl glycosides could not be identified due to insufficient material.

Conclusions

Sedimentation equilibrium and gel filtration analyses indicated that the urine and H.Ep.2 epitectins are polydisperse with average molecular weight 0.9×10^{6} to 1.5×10^{6}. Since these values are probably the most reliable that can be obtained for a heavily glycosylated molecule, the SDS-PAGE values of 350 000 to 400 000 reported by us and other investigators [1, 5, 11, 12, 13, 17, 21, 50] for the MUC1 glycoprotein probably represent monomeric units. Three to four of these units apparently self-associate to give the native molecules. It is possible that a combination of hydrophobic as well as carbohydrate-carbohydrate interactions similar to those reported for other cell surface glycoconjugates [52, 53] could contribute to the association/aggregation of the epitectin molecules. Thus, in solution this smaller glycoprotein will have physicochemical characteristics similar to those of the epithelial mucins having molecular sizes ranging from 2 to 40×10^{6} [54]. The same hydrophobic forces could also be responsible, at least partly, for the observed interaction of epitectin with the co-chromatographing low molecular weight glycoproteins.

The neutral saccharides of epitectin from both urine and H.Ep.2 cells contain three common structures, namely Gal1 → 3 GalNAc(OH); GlcNAc1 → 6 (Gal1 → 3) GalNAc(OH) and Gal1 → 4 GlcNAc1 → 6 (Gal1 → 3) GalNAc(OH). Unfortunately, the quantity of the acidic oligosaccharide alditols was insufficient for the elucidation of the structure of the individual components by FAB/MS. Therefore, these were only identified tentatively by their elution profiles on gel filtration systems (Table 2). It should be noted that in both neutral and acidic oligosaccharides only the core 1 and 2 structures are present. The presence of some Asn-linked saccharides in epitectin was established by mannose labeling and *N*-glycanase susceptibility. Thus, at least qualitatively, the carbohydrate structures in urine epitectin produced by normal differentiated bladder epithelial cells are surprisingly similar to those in epitectin produced by the malignant H.Ep.2 cells.

The remarkable similarities in the total carbohydrate content, the carbohydrate composition and structures of saccharides between epitectin from urine, a non-malignant source, and H.Ep.2 cells is very surprising. These findings do not support the suggestions that all MUC1 glycoproteins from cancer cells are underglycosylated compared to those produced by normal cells [5, 6, 10, 21]. There is also no evidence for the presence in human urine epitectin of polylactosaminoglycan structures similar to those found in the MUC1 glycoprotein isolated from human milk, another non-malignant source [9].

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