Synthesis and Intracellular Trafficking of Muc-1 and Mucins by Polarized Mouse Uterine Epithelial Cells*

(Received for publication, April 30, 1996, and in revised form, July 29, 1996)

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Mucins function as a protective layer rendering the apical surface of epithelial cells nonadhesive to a variety of microorganisms and macromolecules. Muc-1 is a transmembrane mucin expressed at the apical cell surface of mouse uterine epithelial cells (UEC) that disappears as UEC become receptive for embryo implantation (Surveyor, G. A., Gendler, S. J., Pemberton, L., Das, S. K., Chakraborty, I., Julian, J., Fimental, R. A., Wegner, C. W., Dey, S. K., and Carson, D. D. (1995) Endocrinology 136, 3639–3647). In the present study, the kinetics of Muc-1 assembly, cell surface expression, release, and degradation were examined in polarized mouse UEC in vitro. Mucins were identified as the predominant glycoconjugates synthesized, apically expressed, and vectorially released in both wild-type and Muc-1 null mice. When mucins were released, greater than 95% were directed to the apical compartment. Approximately half of the cell-associated mucins lost during a 24-h period were found in the apical compartment. Vectorial biotinylation detected apically disposed, cell-surface mucin and indicated that at least 34% of these mucins are released apically within 24 h. This suggests that release of mucin ectodomains is part of the mechanism of mucin removal from the apical cell surface of UEC. Half-lives of total cell-associated mucins and Muc-1 were 19.5 ± 1 and 16.5 ± 0.8 h, respectively. Muc-1 represented approximately 10% of the [3H]glucosamine-labeled, cell-associated mucins. Studies of the kinetics of intracellular transport of Muc-1 indicated transit times of 21 ± 15 min from the rough endoplasmic reticulum to Golgi apparatus and 111 ± 28 min from the Golgi apparatus to the cell surface. Collectively, these studies provide the first comprehensive description of Muc-1 and mucin maturation, metabolism, and release by polarized cells, as well as defining a major metabolic fate for mucins expressed by UEC. Normal metabolic processing appears to be sufficient to account for the removal of Muc-1 protein during the transition of UEC to a receptive state.

Mucins are a heterogenous class of large molecular weight glycoproteins (apparent Mr of 3 × 10^{5}–10^{7}) that function in vertebrates to protect epithelial cell surfaces from pathogens and digestive enzymes as well as to lubricate these surfaces (1–4). Greater than 50% of mucin dry weight consists of oligosaccharides that are covalently linked to hydroxy amino acids through an α,3 linkage to an N-acetylgalactosamine residue (5). Mucin oligosaccharide assembly is primarily localized to the Golgi apparatus (6, 7). The heterogeneity observed for a given protein core arises from the variable number of tandem repeats in a given gene (allelic polymorphism) as well as the variable size of the constituent oligosaccharides and degree of glycosylation (3, 8). The highly glycosylated, extended structures of mucins produce a large hydration sphere upon which the viscoelastic properties of secreted mucins and the proposed masking function of transmembrane mucins are dependent (4, 9).

The first mucin to be cloned from humans was the tumor-associated antigen, MUC1 (10, 11). MUC1 is a transmembrane mucin expressed by a large number of simple secretory epithelial cells and aberrantly expressed in an underglycosylated form by cancer cells (3, 8, 12). Mucins, in general, and MUC1, in particular, are expressed in a polarized manner by normal epithelia. However, it is unclear whether these processes differ between normal and cancer cells or it is a function of cell polarity. MUC1 has been proposed to function as an anti-adhesive molecule in both cadherin- and integrin-mediated cell adhesion events in vitro (12, 13). A correlation has been observed between the metastatic potential of primary tumors and the degree of glycosylation on MUC1 ectodomain (1, 3, 14). The mouse homolog, designated Muc-1, has a high degree of homology and amino acid identity to the human sequence in the transmembrane and cytoplasmic tail domains but not in the extracellular domain. Moreover, the mouse homolog does not display the allelic polymorphism that characterizes the human homolog (15). In Muc-1 null mice, the growth rates of primary breast tumors were significantly slower than in the wild-type strain, although there was no significant difference in the tumor metastasis (16). Alternatively, MUC1 may facilitate metastasis by serving as a ligand for E- and P-selectin-mediated adhesion processes (17–19). In addition, MUC1 has been proposed to protect carcinomas from destruction by the immune system (20, 21).

The apical cell surface of luminal uterine epithelial cells (UEC) mediates the initial phases of embryo-uterine interac-

1 The abbreviations used are: UEC, uterine epithelial cells; BPA, Bousinia parapura lectin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonate; D-PBS, Dulbecco’s phosphate-buffered saline; D-PBS-CM, Dulbecco’s phosphate-buffered saline plus calcium and magnesium; DBA, Dolichos biflorus lectin; DSA I, Datura stramonium (I) lectin; GS-I, Griffonia simplicifolia (I) lectin; HBS buffer,-buffered saline solution; HPA, Helix pomatia lectin; MPA, Macula pomifera lectin; N-glycosidase, peptidase; N-glycosidase A; polyacrylamide gel electrophoresis; PIMS, protease inhibitor mixture solution; PNA, Arachis hypogaea lectin; Pronase, Streptomyces griseus protease; PWN,
tions during early pregnancy, i.e. implantation (22). The apical cell surface of UEC is only permissive for embryo attachment during a well defined but transient period termed the "window of receptivity" (23). Studies from our lab have identified Muc-1 as one glycoconjugate expressed at the apical cell surface of UEC (24). Furthermore, this expression is markedly reduced during early pregnancy coinciding with the general reduction of the glycoalyx (24, 25). It has been proposed that Muc-1 functions as an anti-adhesive molecule during the peri-implantation period in mice by masking embryo attachment sites (24). Thus, the transition to a "receptive" uterine state is proposed to be, in part, contingent upon Muc-1 removal. Understanding the kinetics of Muc-1 maturation and removal is necessary to develop a detailed understanding of Muc-1 expression by UEC. In this study, we have utilized polarized mouse UEC cultured in vitro to estimate the rate of Muc-1 protein clearance as well as the kinetics of intracellular movement of Muc-1 to the apical cell surface. In addition, we demonstrate that mouse UEC express other mucins, that these other mucins are associated with the apical cell surface, and that their rate of protein clearance is similar to that of Muc-1. In general, apical release is a major metabolic fate of apically disposed, cell-associated mucins of UEC.

**EXPERIMENTAL PROCEDURES**

**Materials—**Female mice (CF-1) were purchased from Sasco (Omaha, NE) and Muc-1 null female mice (129SvJxC57BL6) were generated as described previously (16). Tissue culture media components were obtained from Irvine Scientific (Santa Ana, CA) and Life Technologies, Inc. [35S]Methionine (translation grade, 1,000 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA) or ARC Inc. [3H]Glucosamine (50 µCi/ml) was purchased from Duolbecco's phosphate-buffered saline (D-PBS) containing 0.1% (w/v) sodium azide at 4°C for 10 min. Excess D-PBS was removed from the filter, and 100 µl of 98% isopropanol extraction buffer (0.5% (w/v) Nonidet P-40 and 2 mM EDTA, pH 8.0, in D-PBS at pH 7.0) was added at 4°C. After 5 min of incubation at room temperature the extraction buffer was removed and 100 µl of room temperature extraction buffer was applied and removed immediately. Sodium azide and protease inhibitor mixture (PIM) were added to the pooled cell-associated Nonidet P-40 extract to give a final concentration of 0.02% (w/v) and 1:100 (v/v), respectively. PIM was composed of 10 mg/ml benzamidine, 2 mM N-ethylmaleimide, 1 mM leupeptin, 1 mM chymostatin, 1 mM pepstatin, and 7.6 trypsin-inhibiting units/ml aprotinin in 0.9% (w/v) NaCl, and 0.9% (w/v) benzyl alcohol. The extracts were centrifuged at 15,000 × g for 40 min at 4°C and used for further analyses.

**Immunoprecipitation of Muc-1—**Primary cultures of UEC were cultured, labeled, washed, and extracted as described above. After preclearing by centrifugation, the extracts were incubated by constant rotary agitation overnight (16–18 h) at 4°C with polyclonal antibody CT-1 at a 1:25 ratio of serum to extract. The rabbit antibody is directed against a synthetic peptide corresponding to the terminal 17 amino acids of the C terminus of Muc-1 and has been described previously (24). The antigen-antibody complex was incubated for 4 h at room temperature with constant rotary agitation after the addition of 64 µl of approximately a 50% slurry of protein A-agarose. The pellet resin was washed twice at room temperature by resuspension and centrifugation at 8,000 × g for 3 min with 1 ml of extraction buffer and twice with 1 ml of D-PBS to remove non-resin-bound material. The immunoprecipitated Muc-1 was solubilized from the resin with addition of 50 µl of SEB. Glycoprotein intrinsic to 90% of the resin was demonstrated by rotating agarose suspensions purchased from E.Y. Labs (San Mateo, CA). All other reagents were purchased from Sigma. All chemicals used were reagent grade or better.

**Polarized Uterine Epithelial Cell Cultures—**Primary cultures of polarized uterine epithelial cells (UEC) were prepared from excised uteri of randomly cycling CF-1 mice as described previously, using Matrigel-coated Anocell 10 culture inserts and culture in serum-free medium (26). Routine staining of such cultures for the epithelial marker proteins, cytokeratins and uromucin, demonstrated that they consisted of >95% epithelial cells.

**Metabolic Labeling—**UEC cultures were established for 18 h prior to metabolic labeling with [3H]glucosamine (50 µCi/ml) in serum-free medium. Preliminary experiments demonstrated that labeling of the cell-associated material reached equilibrium by 8–10 h. At the end of the labeling time, cells were rinsed several times with medium prewarmed to 37°C without label, and the medium as well as cell-associated glycoconjugate fractions were processed for glycoconjugate analysis. The residual material, left after extraction with Nonidet P-40, was extracted with sample extraction buffer (SEB) (0.05 M Tris, pH 7.0, 8 µl urea, 1.0% (w/v) phenylmethylsulfonyl fluoride, 1.0% (v/v) β-mercaptoethanol). Apically released, [3H]-labeled glycoconjugates were collected and centrifuged (100,000 × g for 1 h at 4°C) to remove cellular debris and desalted on a Sephadex G-50 column (6 × 300 mm) equilibrated in D-PBS. The material eluting in the void volume was retained for further analysis. The material eluting in the included volume was discarded.

**Pulse Labeling Studies—**UEC were cultured for 2 days as described above. Labeling was begun by adding medium prewarmed to 37°C containing 50 µCi/ml. In certain cases, the labeling medium was supplemented with cycloheximide (1 µg/ml). Cycloheximide was added 2 h after initiation of the labeling period, and the incubation was continued for the indicated time prior to harvest. Parallel control cultures were incubated for the same period in medium lacking cycloheximide. In other experiments, UEC were collected after labeling with [3H]glucosamine (50 µCi/ml) for the indicated time and processed for cell surface biochemical analyses.

**Equilibrium Chase Labeling—**UEC were cultured for 18 h as described above. The cultured cells were rinsed several times with serum-free medium prewarmed to 37°C, and the cells were metabolically labeled for 20 h with [3H]glucosamine (50 µCi/ml) in serum-free medium. After being rinsed several times with serum-free unlabeled medium, the cells were incubated in this prewarmed medium for intervals up to 24 h. The medium constituting the apically released fraction was collected at selected intervals and processed as described below for mucin analysis. The filters were placed in 100 ml of Dulbecco's phosphate-buffered saline (D-PBS) containing 0.1% (w/v) sodium azide at 4°C for 10 min. Excess D-PBS was removed from the filter, and 100 µl of 98% isopropanol extraction buffer (0.5% (w/v) Nonidet P-40 and 2 mM EDTA, pH 8.0, in D-PBS at pH 7.0) was added at 4°C. After 5 min of incubation at room temperature the extraction buffer was removed and 100 µl of room temperature extraction buffer was applied and removed immediately. Sodium azide and protease inhibitor mixture (PIM) was added to the pooled cell-associated Nonidet P-40 extract to give a final concentration of 0.02% (w/v) and 1:100 (v/v), respectively. PIM was composed of 10 mg/ml benzamidine, 2 mM N-ethylmaleimide, 1 mM leupeptin, 1 mM chymostatin, 1 mM pepstatin, and 7.6 trypsin-inhibiting units/ml aprotinin in 0.9% (w/v) NaCl, and 0.9% (w/v) benzyl alcohol. The extracts were centrifuged at 15,000 × g for 40 min at 4°C and used for further analyses.

**Lectin Binding Studies—**Lectin binding was monitored by incubating [H]-labeled cell-associated glycoconjugates, in 0.5 ml of 0.5% (w/v) Nonidet P-40 in D-PBS and containing 50 µl of lectin-agarose (approximately a 50% suspension) in the absence or presence of 0.75 µm monosaccharide competitor. Cell debris in the apical media was removed by centrifugation at 100,000 × g for 1 h at 4°C. Approximately 5,000–10,000 dpm of [H]-labeled Nonidet P-40-extractable, cell-associated apically released glycoconjugates or 3,000–5,000 dpm of [H]-labeled Muc-1 was used for these assays except where otherwise indicated. These suspensions were incubated for 2 h at room temperature with constant rotary agitation. The unbound material was removed by centrifugation at 8,000 × g followed by four washes with 1.0 ml of binding buffer. The radioactivity associated with the resin was determined by resuspending the pellet in D-PBS and transferring it to scintillation vials for counting. Specificity of binding to the lectin gel for the labeled glycoconjugates was determined by subjecting the radioactivity associated with binding to the resin in the presence of competitor from the radioactivity associated with binding to the resin in the absence of competitor.

**Analysis of Glycoconjugates—**Apical media were fractionated by binding to WGA agarose as described above. The unbound fraction was used for further processing as described below. The WGA-bound glycoconjugates were eluted by incubating the resin in 0.5 M N-acetyl-d-

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**Phytolacca americana** lectin; **RCA-I, Ricinus communis** (I) lectin; **SEB**, sample extraction buffer; **STA**, *Solanum tuberosum* lectin; **WGA**, wheat germ agglutinin from *Triticum vulgaris*. 
glucosamine in D-PBS containing PICS (1:100) and 2 mM EDTA at 25 °C for 4 h. After pelleting the resin, the WGA-bound extract was saved and processed as described below. Recoveries from fractionation of the apical media were 85–90%. Cells were washed several times with D-PBS, precooled for 10 min in D-PBS at 4 °C containing 0.1% (w/v) sodium azide, then extracted with Nonidet P-40 according to the immunoprecipitation protocol. The residual unextracted cell fraction was solubilized in 8 M guanidine hydrochloride, 0.5% (w/v) CHAPS, 20 mM Tris acetate, pH 7.0, 2 mM EDTA, and PIMS (1:100). The pooled Nonidet P-40 extract was processed for immunoprecipitation. The nonimmunoprecipitable Nonidet P-40 extract was collected and processed as described below. The immunoprecipitated Muc-1 was eluted from the protein A by two sequential incubations for 3 h at 25 °C using 8 M guanidine hydrochloride, 0.5% (w/v) CHAPS, 20 mM Tris acetate, pH 7.0, 0.05 M sodium azide, and 2 mM EDTA. This extract was dialyzed and dried under rotary vacuum with the other samples. Both fractions were analyzed by Superose 12 chromatography as described.

The final third of the glycoconjugate fractions was subjected to mild digestion of glycans by pronase digestion by incubating heat-denatured (95 °C for 2 min) samples at 50 °C for 24 h with 1.5 mg/ml predigested Pronase in 0.1 M Tris-HCl, pH 8.0, 2 mM CaCl₂, and 5% (v/v) ethanol buffer. The incubation was continued for an additional 24 h with a freshly prepared addition of predigested Pronase. Predigested Pronase was made by incubating for 30 min at 50 °C 10 mg/ml Pronase in 0.1 M HCl, pH 5.0, 2 mM CaCl₂, and 5% (v/v) ethanol buffer. Pronase-digested samples were concentrated as described above. The third final of the glycoconjugate fractions was subjected to mild alkaline hydrolysis (β-elimination) by incubating the glycoconjugates in 0.1 M NaOH, 0.25 M NaBH₄ at 37 °C for 48 h (29). Samples were neutralized with acetic acid and dried under rotary vacuum prior to analysis. Macromolecules were fractionated under dissociative conditions by molecular exclusion liquid chromatography as described previously (30). Recoveries from this procedure ranged between 85 and 95%.

Biotinylation of Apically Disposed Glycoconjugates—Polarized UEC were collected at the indicated time of incubation with [³H]glucosamine and processed for vectorial biotinylation (31). After aseptically washing the cells three times with 4 °C D-PBS supplemented with 2 mM CaCl₂ and 2 mM MgCl₂ (D-PBS-CM), the cells were incubated at 4 °C for 30 min in 100 μl of D-PBS-CM supplemented with 0.02% (w/v) sodium metaperiodate on the apical surface only and with 500 μM (v/v) ethanol containing 200 mM sucrose. Filters were washed twice with 4 °C Hanks’ balanced salt solution (HBSS) containing 0.1% (w/v) sodium azide. The cells were incubated for 2.5 h at 4 °C under subdued lighting with 500 μl of HBSS containing 0.1% (w/v) sodium azide, apically and basally. Aldehydes formed by periodate oxidation were reacted with 100 μg/ml bovine hyaluridase in HBSS in the apical compartment. The reaction was terminated by washing with HBSS and Muc-1 was immunoprecipitated as described above. Apically disposed and nonimmunoprecipitable cell-associated glycoconjugates were isolated as described above. Biotynlated components in each fraction were selected by incubation with 65 μl of streptavidin-agarose (approximately a 50% suspension) for 4 h at room temperature. The streptavidin-agarose was washed four times with 1 ml of binding buffer. The radioactivity associated with the resin was determined by resuspending the pellet in 400 μl of binding buffer and transferring it to scintillation vials for counting. Total cell-associated Muc-1 was determined by immunoprecipitation as described above. Radioactivity associated with apically disposed Muc-1 was defined as that portion of the immunoprecipitable [³H]-Muc-1 that bound to streptavidin-agarose. Cell viability at the end of the incubation with periodate and biotinylation exceeded 90% as indexed by morphology and trypan blue dye exclusion. Cells remained viable by these criteria for at least 24 h following these treatments.

Other Procedures—Protein concentrations were determined by the procedure of Lowry et al. (32) using bovine serum albumin as a standard.
Muc-1 was digested with peptide: N of mucin oligosaccharides. A greater proportion of apically re-actylgalactosamineresidues (33), consistent with the presence and sensitivity to mild alkaline hydrolysis (used Pronase sensitivity to identify protein-linked oligosaccharides chemically analyzed for the presence of mucins. The approach on macromolecules were extracted with Nonidet P-40 and biochemically identified as mucins. These data further suggested that WGA-binding mucins are a major component of the apically released, \(^{3}H\)glucosamine-labeled glycoconjugates in the Nonidet P-40 extract. It was concluded that the immunoprecipitation for Muc-1 was specific and efficient.

**Lectin Binding Studies**—As shown in Table I, the lectin-binding characteristics of immunoprecipitated, cell-associated Muc-1 were similar to those of the other glycoconjugate fractions. Binding to WGA was the highest, followed by BPA, RCA-1, and *Ulex europaeus* (I) lectin. BPA recognizes \(\beta\)-N-acetylgalactosamine residues (33), consistent with the presence of mucin oligosaccharides. A greater proportion of apically released components bound to *Canavalia ensiformis* lectin than in the cell-associated fraction. Since *C. ensiformis* lectin primarily recognizes \(\alpha\)-linked structures (34), these results suggest an enrichment in \(\alpha\)-linked glycoconjugates in the apical secretions. The enrichment was paralleled by a decrease in BPA binding relative to the cell-associated glycoproteins. It was concluded that the lectin-binding characteristics of the cell-associated and apically released glycoconjugates were consistent with their identification as mucins. These data further suggested that WGA-binding mucins are a major component of the apically released, \(^{3}H\)glucosamine-labeled glycoconjugates.

**Identification and Characterization of Cell-associated Glycoconjugates of Polarized UEC**—Cell-associated, radiolabeled macromolecules were extracted with Nonidet P-40 and biochemically analyzed for the presence of mucins. The approach used Pronase sensitivity to identify protein-linked oligosaccharides and sensitivity to mild alkaline hydrolysis (\(\beta\)-elimination) to identify mucin oligosaccharides. Intact Muc-1 had a hydrodynamic radius greater than that of a dextran standard of median \(M_r = 531,000\) (Fig. 2A); however, mild alkaline hydrolysis quantitatively shifted the radioactive components to a broad peak of lower \(M_r\) forms with a median hydrodynamic radius of 12,000 relative to dextran standards (\(K_v = 0.7\). A small portion of the Muc-1 radioactivity (6%) did not shift following mild alkaline hydrolysis suggesting the presence of a small amount of \(\alpha\)-linked structures. Consistent with these results, very little shift in \(M_r\) was observed when Muc-1 was digested with peptide:N-glycosidase (data not shown). Furthermore, this material was insensitive to endo-\(\beta\)-galactosidase digestion, suggesting a lack of lactosaminoglycans in these structures (data not shown). As shown in Fig. 2B, intact \(^{3}H\)glucosamine-labeled, non-Muc-1 glycoconjugates in the Nonidet P-40 extract displayed a heterogeneous elution profile with approximately 76% of these glycoconjugates co-eluting with a \(K_v \approx 0.1\). Following mild alkaline hydrolysis, almost all of the radioactivity migrated with a \(K_v \approx 0.7\) similar to that of Muc-1 oligosaccharides. Pronase digestion converted a majority (78%) of the material to a slightly larger size distribution than the oligosaccharides released by mild alkaline hydrolysis. A minor Pronase-resistant fraction (22%) also was present. Similar results were obtained for \(^{3}H\)glucosamine-labeled, cell-associated glycoconjugates from Muc-1 null UEC (mucins comprising approximately 74%) (Fig. 2C). Very little shift in \(M_r\) was observed when the non-Muc-1 glycoproteins were digested with peptide:N-glycosidase, indicating a small fraction of \(\alpha\)-linked structures on these macromolecules (data not shown). Collectively, these data indicated that mucins, including Muc-1, are the major (76%) glycoproteins expressed by UEC.

**Identification and Characterization of Secreted Glycoconjugates**—As shown in Fig. 3, the vast majority (>90%) of the secreted glycoconjugates were recovered in the apical compartment. Furthermore, most (75%) of the glycoconjugates in either apical or basal secretions bound to WGA. No components were specifically immunoprecipitated with the Muc-1 endodomain antibody suggesting that little, if any, intact Muc-1 was released into the apical compartment (data not shown). Further analyses revealed other distinctions among these fractions. Intact WGA-binding glycoconjugates eluted as a heterogeneous population with a similar size distribution as the cell-associated, non-Muc-1 mucins (Fig. 4A). Mild alkaline hydrolysis shifted most (84%) of the radioactive components to a broad peak of smaller size forms with a median hydrodynamic radius similar to that observed for the cell-associated mucin oligosaccharides (compare with Fig. 2B). A minor fraction (5%) was resistant to mild alkaline hydrolysis suggesting that these glycoconjugates contained a small proportion of \(\alpha\)-linked structures. Pronase digestion of this material released a large fraction (55%) eluting as slightly larger structures than the products of mild alkaline hydrolysis; however, a Pronase-resistant portion (25%) retained the same size characteristics as the undigested material. As shown in Fig. 4B, the intact non-WGA-binding glycoconjugates eluted as a smaller heterogeneous population than the WGA-binding glycoconjugates. Both mild al-
Kaline hydrolysis and Pronase digestion shifted a large portion (68%) of the radioactive components to a broad peak of lower Mr forms similar to those of the WGA-binding glycoconjugates released by alkaline hydrolysis. Similar results were obtained when secreted glycoconjugates from UEC of Muc-1 null mice were analyzed demonstrating that most of these components were not derived from Muc-1 (Fig. 5). Consistent with their high degree of sensitivity to mild alkaline hydrolysis, very little shift in Mr was observed when these glycoproteins were digested with peptide-N-glycosidase (data not shown). Collectively, these data demonstrate that mucins are the predominant (78%) apically released glycoconjugates of polarized UEC.

The intact basally released glycoconjugates of wild-type UEC were much smaller than the majority of the apically released glycoconjugates and eluted with median hydrodynamic radii of Mr 5 x 10,000 relative to dextran standards (Kav 5 0.78) (Fig. 6A). Mild alkaline hydrolysis marginally shifted the size distribution of these components to a median Kav = 0.87. Pronase digestion of this material had a more pronounced effect and shifted the distribution of most glycoconjugates to the fully included volume. Peptide-N-glycosidase also converted a fraction (12%) of these glycoconjugates to a form migrating at or near the fully included column volume and indicated the presence of N-linked structures (data not shown). Collectively, these data indicated that the basally secreted glycoconjugates were biochemically distinct from the WGA-binding, apically secreted glycoconjugates. Similar results were obtained when [3H]glucosamine-labeled basalsecretionsfromMuc-1nullUEC were analyzed indicating that these components were not Muc-1 derived (Fig. 6B).

**Metabolic Half-life and Kinetics of Apical Mucin Release**—To determine the metabolic fate of UEC mucins, polarized cells were metabolically labeled to equilibrium with [3H]glucosamine and then a chase period was initiated. The quantity of cell-associated mucins at a given time point was determined as described above. As shown in Fig. 7A, loss of [3H]-labeled mucins, including Muc-1, began immediately upon initiation of the chase. The metabolic half-life for Muc-1 was 16.5 ± 0.8 h. The mean metabolic half-life for non-Muc-1 mucins was determined to be 19.5 ± 1 h and was not statistically different from

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**FIG. 2.** Superose 12 molecular exclusion chromatography of cell-associated glycoconjugates. UEC were metabolically labeled for 24 h with [3H]glucosamine and extracted, and glycoconjugates were isolated as described under “Experimental Procedures.” Panel A, the immunoprecipitated Muc-1 was analyzed as intact (●) or digested by mild alkaline hydrolysis (◇) as described under “Experimental Procedures.” Panel B, Nonidet P-40-extractable components depleted of Muc-1 were analyzed as intact (●), mild alkaline-hydrolyzed termed β-elimination (◇), or Pronase-digested (○). Panel C, Nonidet P-40-extractable components of Muc-1 null mice were analyzed as intact (●), mild alkaline-hydrolyzed termed β-elimination (◇), or Pronase-digested (○). The elution positions of the following molecular mass markers are indicated at the top of the figure: V_o, blue dextran (Mr > 2 x 10^6); 1, 531-kDa dextran; 2, 151-kDa dextran; 3, 71-kDa dextran; 4, 40-kDa dextran; 5, commercial [3H]heparin (median molecular mass of 10 kDa); and V_t, potassium dichromate.

**FIG. 3.** [3H]Glucosamine-labeled, WGA-binding glycoproteins are preferentially released apically by UEC. UEC were metabolically labeled for 24 h with [3H]glucosamine. Apical and basal media were collected, and total glycoproteins in these fractions were fractionated by WGA affinity chromatography as described under “Experimental Procedures.” The values that are shown are the mean ± S.D. for determinations performed on three separate filters. For the WGA bound (+) apical medium sample, the standard deviation of the mean is less than 2.5% and is hidden by the filled bar. Symbols: +, WGA binding performed in the presence of 0.5 M N-acetylglucosamine; –, WGA binding performed in the absence of N-acetylgalactosamine.
that of Muc-1. Muc-1 represented approximately 9% of the total [3H]glucosamine-labeled cell-associated mucins produced at all time points. It was concluded that the half-life of Muc-1 was representative of bulk mucins expressed by UEC.

The kinetics of apical mucin release was studied to determine if apical release was a major metabolic fate of UEC mucins. Radiolabeled mucins appeared in the medium with no apparent lag period and continued to accumulate over the 24-h chase period (Fig. 7B); however, during this 24-h period only about 50% of the mucins lost from the cell-associated fraction were recovered in the apical media. Therefore, it seems likely that the remainder of the mucins are degraded either inside of the cell or after release to the media. Supporting this interpretation was the observation that the accumulation of low Mr [3H]-labeled products in the media more than accounted for the loss of the glycoconjugates from the cell-associated fraction. Based on the specific activity of the glucosamine precursor, it was estimated that UEC lost at least 2–3 ng of mucin/mg of cellular protein over a 24-h period.

Studies were performed to determine if lysosomal degradation was a major metabolic pathway for Muc-1 and non-Muc-1 mucins in UEC; however, approximately 20% of the cells were lost within 9 h of initiation of treatment with chloroquine even at concentrations 4-fold lower than that used in previous, shorter-term studies on UEC (30). In an attempt to determine if proteolysis was part of the mechanism of Muc-1 and non-

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**Fig. 4.** Superose 12 molecular exclusion chromatography of apically released glycoconjugates of wild-type UEC. UEC glycoconjugates metabolically labeled for 24 h with [3H]glucosamine and released into the apical compartment were collected and were subjected to WGA affinity chromatography as described under “Experimental Procedures.” Panel A, the apically released, WGA-binding components were analyzed as intact (■), digested by mild alkaline hydrolysis (●), or Pronase-digested (○) as described under “Experimental Procedures.” Panel B, the apically released, non-WGA-binding components were analyzed as intact (■), mild alkaline-hydrolyzed (●), and Pronase-digested (○). The molecular mass markers are described in the legend to Fig. 2.

**Fig. 5.** Superose 12 molecular exclusion chromatography of apically released glycoconjugates of Muc-1 null UEC. Muc-1 null UEC glycoconjugates metabolically labeled for 24 h with [3H]glucosamine and released into the apical compartment were collected and were subjected to WGA affinity chromatography as described under “Experimental Procedures.” Panel A, the apically released, WGA-binding components were analyzed as intact (■), digested by mild alkaline hydrolysis (●), or Pronase-digested (○) as described under “Experimental Procedures.” Panel B, the apically released, non-WGA-binding components were analyzed as intact (■), mild alkaline-hydrolyzed (●), and Pronase-digested (○). The molecular mass markers are described in the legend to Fig. 2.
Muc-1 mucin loss from UEC, a protease inhibitor mixture and EDTA were used to inhibit various classes of proteases (30). The maximum inhibition of glycoconjugate loss from the cell-associated fraction observed in the presence of the protease inhibitor mixture for Muc-1 and the other cell-associated mucins was 8% (data not shown). In addition, EDTA treatment caused cell detachment within 1 h making use of this metalloprotease inhibitor not feasible for extended periods (data not shown). Consequently, other approaches were used to determine the fate of apically disposed mucins, including Muc-1.

**Intracellular Movement of Muc-1**—The kinetics of assembly and transport to the cell surface in UEC was examined. Assembly of mucin oligosaccharides on the protein core was used as an index of arrival in the Golgi apparatus (6, 7). First the kinetics of transport of Muc-1 core proteins from their site of synthesis in the rough endoplasmic reticulum to the primary site of O-glycosylation in the Golgi apparatus was studied. Cycloheximide was used to arrest de novo synthesis of Muc-1 protein cores. Preliminary studies had determined that 1 μg/ml cycloheximide inhibited greater than 90% of protein synthesis within 5 min of addition (data not shown). Furthermore, no cell loss was detectable. Cells were prelabeled for 2 h with [3H]glucosamine prior to cycloheximide addition. A lag phase was consistently observed during the first 2 h of labeling, e.g. see Figs. 8 and 9, presumably representing the time required for...
[3H]glucosamine to be transported, incorporated, and equilibrated into sugar nucleotide pools. Therefore, these experiments were not initiated until the linear phase of synthesis, i.e., 2–10 h of [3H]glucosamine addition, had begun. The time required for cessation of [3H]glucosamine incorporation into Muc-1 following cycloheximide addition was considered to be equal to the maximum time required for all preformed Muc-1 core proteins to move from the rough endoplasmic reticulum and be fully glycosylated in the Golgi apparatus, i.e., maximum transit time (Fig. 8). Muc-1 synthesis in untreated control cells was linear throughout the duration of the experiment. The mean transit time and 95% confidence intervals determined were 21 ± 5 min. The values shown are the mean ± S.D. of values obtained for determinations performed on three separate filters in each case. The straight arrow indicates the point at which cycloheximide was added.

The kinetics of Muc-1 movement to the apical cell surface from the Golgi apparatus also was studied. Again, oligosaccharide addition was considered to represent arrival in the Golgi apparatus. The time at which [3H]glucosamine-labeled Muc-1 became accessible to impermeant biotinylation reagents added to the apical compartment was used to estimate the time of arrival at the apical cell surface. Thus, as a function of labeling time, the difference in time between arrival in the Golgi apparatus and the time of appearance at the apical cell surface should reflect the transit time from the Golgi to apical cell surface. Vectorial labeling reagents that reacted with protein cores, e.g., lactoperoxidase-catalyzed radiodination or amino group-directed biotinylation, were found to be much less efficient than the carbohydrate-direct biotinylation procedure used. In preliminary experiments, it was determined that 35–40% of radiolabeled Muc-1 was not accessible to biotinylation under optimal biotinylation conditions in UEC labeled to equilibrium with [3H]glucosamine. Thus, the slope of the line defining kinetics of arrival at the apical surface is expected to be proportionately shallower than that of the line defining the linear phase of total Muc-1 synthesis. The inaccessible fraction may reflect the presence of an intracellular pool of Muc-1 or cell surface forms that are otherwise inaccessible to or nonreactive with the biotinylation reagents. As shown in Fig. 9, [3H]glucosamine incorporation into total cell-associated Muc-1 became linear approximately 2 h after addition of [3H]glucosamine and remained linear for at least 8 h. Detection of [3H]glucosamine-labeled Muc-1 at the apical cell surface appeared to be biphasic with amounts marginally above background levels detected during the first 3 h; however, substantial detection first occurred between 3.5 and 4 h of the labeling period. Extrapolation of these lines to the intercept indicated a difference of 111 ± 28 min between the time at which [3H]glucosamine-labeled Muc-1 could first be detected in the cell and its arrival at the apical cell surface.

Collectively, the data indicated that the maximum intracellular transit time of Muc-1 from initiation of synthesis in the rough endoplasmic reticulum to the Golgi was 21 ± 15 min, and the minimum transit time from the Golgi apparatus to the plasma membrane was 111 ± 28 min. Therefore, the intracellular transit time for Muc-1 from the endoplasmic reticulum to the cell surface was approximately 2.2 ± 0.7 h.

Apically Disposed, Cell-surface Mucins Are Released into the Apical Compartment—It was of interest to determine if apically disposed mucins were released from UEC as one aspect of their metabolism. The fate of apically disposed mucins accessible to biotinylation was monitored. UEC were metabolically labeled to equilibrium and subsequently biotinylated on the apical cell surface. The fate of these biotinylated components was assessed initially and after 24 h of chase (Table II). Approximately 75 and 85% of total cell-associated biotinylated mucins and Muc-1, respectively, were lost during the 24-h chase period. At least 34% of the apically disposed biotinylated mucins were released to the apical compartment during this period. Collectively, these experiments demonstrate that apical
release of apically disposed mucins is one major metabolic fate for these mucins.

**DISCUSSION**

A series of studies were performed to monitor aspects of assembly, intracellular transport, and secretion of Muc-1 and other mucins in polarized UEC. A model based on these results is summarized in Fig. 10. The maximum transit time for Muc-1 from the site of core protein synthesis in the rough endoplasmic reticulum to the site of glycosylation in the Golgi complex was approximately 20 min. Lactosaminoglycans as well as heparan sulfate proteoglycans have a similar transit time in UEC (30, 35). In addition, MUC1 expressed by human carcinomas displays a similar rough endoplasmic reticulum to Golgi transit time (36, 37). In contrast, Muc-1 transit from the Golgi to the cell surface is significantly longer (approximately 111 min) than that of either lactosaminoglycans (30 min) or proteoglycans (6 min) synthesized by UEC (30, 35) or MUC1 in human carcinomas (36–38). Transport of glycoproteins to the cell surface from the endoplasmic reticulum has been suggested to depend on the rate of transport from both the endoplasmic reticulum and Golgi (39). The similarity in transit times, from endoplasmic reticulum to the Golgi apparatus (approximately 30 min) for mucins, N-linked lactosaminoglycans, and proteoglycans synthesized by UEC indicates that these cells do not discriminate among glycoconjugates at this aspect of intracellular transport. Rather, processing or retention factors affecting transit time from the Golgi to the cell surface appear to be the dominant factors affecting intracellular transport kinetics among these three classes of glycoconjugates. Most of the assembly steps in synthesis of all three glycoconjugate classes occur in the Golgi apparatus; however, distinct sets of enzymes are involved. Thus, the differences in rates of Golgi to cell surface transit between mucins and the other glycoprotein classes in UEC may reflect kinetic differences in these glycosylation reactions.

Aberrant glycosylation may affect intracellular transit times in tumor cells. For example, MUC1 expressed by human carcinomas is underglycosylated, and the residency time in the endoplasmic reticulum of MUC1 in the MCF-7 breast carcinoma cell line is approximately 45 min (37). In addition, other underglycosylated mucins expressed by carcinomas display shorter transit times to the cell surface than observed for Muc-1 in normal UEC (40, 41). Thus, the current studies further support the notion that intracellular transit time is proportional to the degree of protein glycosylation in the Golgi apparatus.

The metabolic half-lives of 16.5 ± 0.8 h for cell-associated Muc-1 and 19.5 ± 2.0 h for cell-associated non-Muc-1 mucins are not statistically different. In previous studies, the metabolic half-life of lactosaminoglycans and proteoglycans in UEC were determined to be 6 and 2 h, respectively (30, 35). Thus, mucins, including Muc-1, have a long half-life compared with other glycoproteins expressed by UEC. It has been suggested that the biological half-life of a protein is influenced by the quantity and size of the oligosaccharides on a glycoprotein (42, 43). Mucin substitutions on a given glycoprotein enhance the half-life by protecting proteins from proteolysis and thus prolong the half-life of these glycoconjugates. Loss of mucins from the cell-associated fraction of UEC appears to reflect both degradative and release processes with at least 50% of the mucins destined for apical release. Accumulation of low M<sub>r</sub> products in the medium accounted for the balance of mucin degradation suggesting degradation pathways as another contributor to mucin loss.

Coexpression of multiple secreted mucin core proteins along with MUC1 has been determined in columnar epithelial cells from a variety of normal tissues by Northern analysis and immunohistochemistry (8, 44). As shown in both wild-type and Muc-1 null mice, UEC express cell-associated mucins in addition to Muc-1. At least two other high molecular weight mucin glycoproteins are expressed by mouse UEC. Northern blotting and immunohistochemical experiments indicate that leukosialin, a transmembrane mucin, is not expressed by mouse UEC, and the identity of non-Muc-1 mucins expressed by mouse UEC remains undetermined.

During early pregnancy, the glycocalyx present on the apical surface of luminal UEC becomes significantly diminished in many species (22). Reduction of both Muc-1 mRNA and protein expression coincides with the loss of the luminal glycocalyx prior to embryonal implantation (24). Mucins, in particular Muc-1, are proposed to function as negative modulators of blastocyst attachment to UEC (24). Muc-1 expression is enhanced by estrogen and inhibited by progesterone, but whether this regulation is direct or indirect is unclear (24). The effects of steroid hormones on Muc-1 expression are similar to those on the synthesis of a variety of glycoproteins in female reproductive
tract tissues (35, 45, 46). Moreover, glycoproteins expressed by UEC have been shown to be metabolized more rapidly in response to estrogen (46). Consequently, hormonal regulation of Muc-1 expression may occur at both transcriptional and post-transcriptional levels. During the 48-h period in which the glyocalyx and Muc-1 are reduced, when Muc-1 mRNA levels are falling, approximately 80–90% of Muc-1 protein is expected to be lost during a 48-h period in the absence of de novo synthesis. Thus, the half-life of Muc-1 is sufficiently short to account for the loss of Muc-1 protein observed during the pre-implantation period. These observations indicate that transcriptional down-regulation rather than changes in Muc-1 stability is primarily responsible for loss of Muc-1.

Acknowledgments—We appreciate the helpful comments and critical reading of this manuscript by M. French, D. Hoke, G. Surveyor, X. Zhou, and Mrs. M. DeSouza, A. Jacobs, S. Liu, and E. G. Regisford. We appreciate the excellent secretarial assistance of Sharron Kingston and graphic designs of Alisha Tiznor and Karen Hensley.

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