N-terminal Cleavage of the Salivary MUC5B Mucin

ANALOGY WITH THE VON WILLEBRAND PROPOPEPTIDE?*

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Sequence similarities between the oligomeric mucins (MUC2, MUC5AC, MUC5B) and the von Willebrand factor suggest that they may be assembled in a similar way. After oligomerization, a fragment corresponding to the D1 and D2 domains is released from the von Willebrand factor. This cleavage does not appear to occur in pig submaxillary mucin, the only mammalian mucin in which this cleavage has been examined thus far, but whether other oligomeric mucins undergo N terminus proteolysis is not known. Antibodies recognizing the D1, D2, D3, and the first Cys domains in MUC5B were established and used to investigate to what extent proteolytic cleavage occurs within the N-terminal part of salivary MUC5B. The antibodies against the D1 and D2 domains identified a polypeptide corresponding in size to a MUC5B fragment generated by cleavage within the D' domain analogously with the von Willebrand factor propolypeptide. The antibodies did not recognize the main mucin population, suggesting that the major part of salivary MUC5B is subjected to this cleavage. An antibody recognizing the D3 domain was used to reveal a second cleavage site in the “soluble” but not in the “insoluble” MUC5B fraction: the first structural difference observed between soluble and insoluble salivary MUC5B. The identification of these cleavage events shows that the N-terminal sites for MUC5B oligomerization are present in the D3 domain and/or in domains located C-terminal to this part of the molecule.

The genes for the MUC2, MUC5AC, MUC5B, and MUC6 mucins are clustered on chromosome 11p15.5 (1), and these glycoproteins have all been shown/predicted to be large, secreted, oligomeric mucins (2–5). The MUC2, MUC5AC, and MUC5B mucins show a high degree of similarity in the N- and C-terminal ends to the cysteine-containing D domains of the von Willebrand factor (vWF) (6–8). The N-terminal propolypeptide of the vWF comprising the D1 and D2 domains is cleaved after oligomerization (9); however, this cleavage does not appear to occur in porcine submaxillary mucin (PSM), the only mammalian mucin in which this process has been examined so far (10). Whether or not cleavage occurs in the N terminus of other mucins (such as the human oligomeric ones) is not known. Herrmann et al. (2) have identified a C-terminal cleavage fragment in MUC2, and it has been shown that the C-terminal part of MUC5B may be subjected to proteolytic cleavage, giving rise to at least two different fragments (4). The C terminus of rat Muc2 undergoes cleavage by furin after dimerization (11).

The human oligomeric mucins have been postulated to be assembled in a manner similar to the vWF (6), and PSM has been shown to form C-to-C dimers in the endoplasmic reticulum (12, 13) followed by N-to-N multimerization through the D-domains in the distal part of the Golgi (10). PSM shows homologies to MUC5B, MUC2, and vWF, including the D1, D2, and D3 domains, and it has been suggested that half-cysteine residues in the D1 and D3 domains are needed for multimerization of this mucin (10). However, the assembly of the various mucins may follow dissimilar pathways. For example, MUC2 occurs as an insoluble complex that resists extraction with guanidinium chloride unless disulfide bonds are cleaved (2, 14, 15), and in addition, human salivary MUC5B has been shown to form such insoluble assemblies (16). The formation of insoluble MUC2 and MUC5B is likely to involve “cross-links” in addition to those that join mucin monomers end-to-end into linear structures. Possibly, the non-reducible bonds that (together with disulfide bonds) maintain the integrity of the insoluble MUC2 complex fulfill this function (2), but no such bonds have been identified in MUC5B. The structural difference between soluble and insoluble MUC5B therefore remains to be elucidated.

MUC5B has been identified in respiratory tract, endocervical secretions, and saliva (4, 17) as well as in the colon (18) and gallbladder (18, 19). MUC5B is the first mucin gene in which both the cDNA and genomic sequences are known (8). The structure predicted from the deduced amino acid sequence of the apoprotein fits well with that postulated previously for cervical mucins, now known to be mainly MUC5B, based on biochemical/biophysical studies (20).

Knowledge of the MUC5B apoprotein sequence has allowed us to develop antibodies against the N-terminal domains of MUC5B and to investigate whether this mucin is subjected to proteolytic cleavage events similar to those that occur in vWF. Antibodies against the D1 and D2 domains were used to identify a polypeptide with a size expected for a MUC5B fragment generated via proteolytic cleavage in the D’ domain. The antibodies did not recognize the main populations from either soluble or insoluble MUC5B, suggesting that the major part of salivary MUC5B is subjected to this cleavage. An antibody against the D3 domain revealed a second MUC5B fragment from soluble MUC5B, whereas this antibody reactivity re-
nained associated with the mucin monomers obtained from insoluble MUC5B. The identification of this cleavage event may provide a structural explanation for the difference between soluble and insoluble salivary MUC5B.

**EXPERIMENTAL PROCEDURES**

**Materials**—Guanidinium chloride (practical grade) was from ICN Biochemicals (Costa Mesa, CA). Stock solutions of guanidinium chloride (−8 m) were treated with activated charcoal and filtered through a PM10 filter (Amicon, Beverly, MA) before use. Nitrophenyl phosphate and bovine serum albumin (Fraction V, pH 7.0) were purchased from Serva (Heidelberg, Germany); iodoacetamide and N-ethylmaleimide were purchased from Sigma; diethylpyrocarbonate was purchased from Fluka (Buchs, Switzerland). Biotin hydrazide was from Vector Laboratories Inc. (Burlingame, CA), and alkaline phosphatase conjugated streptavidin was from Roche Molecular Biochemicals. Coverplate immunostaining chambers were from Shandon (Pittsburgh, PA). The horseradish peroxidase- and alkaline phosphatase-conjugated swine anti-rabbit sera, as well as the anti-albumin antibody, StreptABComplex/horseradish peroxidase kit, and the liquid diaminobenzidine substrate-chromogen system, were bought from DAKO (Glostrup, Denmark).

The LUM5B-2 antibody raised against a repetitive sequence flanking the glycosylated domains of the MUC5B mucin has been described previously (4). The ECL Western detection kit, polyclinivlnde diiufro membranes, and the Superose 6 column were from Amer sham Biosciences. Ethanol-fixed human tracheal tissue was a kind gift from Dr. Jacques Bara, 482-INSERM, Paris, France. All other reagents were of A.R. or equivalent quality.

**Preparation of MUC5B Antisera**—Synthetic peptides with sequences 1302YWNHRPPEPGLGGDFD 1313 (LUM5B-5(Cys)) present in the first Cys domain and KAYEDFVNYLRR 1322 (LUM5ACB-2(D1)), 434ARVQACDIVAYSL 1334 (LUM5B-7(D2)), as well as 1131CYDKDGNNYYDVGARYV 1346 (LUM5B-10(D2)) present in the D1, D2, and D3 domains, respectively, of the MUC5B peptide were conjugated to keyhole limpet hemocyanin and used to raise antibodies in rabbits. An initial injection of 100 μg of peptide in Freund’s complete adjuvant given intracutaneously was followed 4 weeks later by a booster injection of 100 μg of peptide in Freund’s incomplete adjuvant. Rabbits were bled 2–3 weeks later, and the antisera were named LUM5B-5(Cys), LUM5ACB-2(D1), LUM5B-7(D2), and LUM5B-10(D3), respectively.

**Preparation of Salivary Mucins**—Human submandibular/sublingual (HSMSL) saliva was collected from three individuals (data from one subject was omitted) and the antisera were named LUM5B-5(1-Cys), LUM5ACB-2(D1), LUM5B-7(D2), and LUM5B-10(D3), respectively.

**Preparation of MUC5B**

**Analysis of Salivary Mucins**—Human submandibular/sublingual (HSMSL) saliva was collected from three individuals (data from one individual are shown), and mucins were isolated as described previously (16). In short, saliva was collected on ice and separated into sol and gel phases by ultracentrifugation. The gel phase was extracted using 6 m guanidinium chloride, 5 m EDTA, and 10 mM sodium phosphate, pH 6.5, and the mucin solution was brought into 8 m guanidinium chloride (Beckman 70.1 Ti rotor, 40,000 rpm, 20 °C for 2 h and 45 min or 8 h). Fractions (300 μl) were collected from the top of the tubes and analyzed for carbohydrate, Aνmax, density, and antibody reactivity.

**Rate-Zonal Centrifugation**—Rate-zonal centrifugation was performed as described (21). Guanidinium chloride gradients (6–8 m) were formed in 14 × 90 mm centrifuge tubes using a Hoefer gradient maker connected to an LKB 2232 Microperpex pump at a flow rate of 50 ml/hour. Samples were layered onto the top of the gradient, and tubes were spun in a Beckman L-70 Optima centrifuge (Beckman SW41 Ti rotor, 40,000 rpm, 20 °C for 2 h and 45 min or 8 h). Fractions (300 μl) were collected from the top of the tubes and analyzed for reactivity with the LUM5B-2, LUM5B-5(Cys), and LUM5B-10(D3) antisera.

**Gel Chromatography**—Gel chromatography was performed on a Superose 6 column eluted with 4 m guanidinium chloride, pH 7, at a flow rate of 0.25 ml/min. Fractions (0.25 ml) were analyzed for reactivity against the LUM5B-7(D2) and LUM5ACB-2(D1) antisera and an antibody against alkaline phosphatase.

**SDS-PAGE and Western Blotting**—Fractions from the Superose 6 column reacting with the LUM5B-7(D2) and LUM5ACB-2(D1) antisera were pooled and dialyzed against water, freeze-dried, dissolved in 62.5 mM Tris/HCl buffer, pH 6.8, containing 2.5% (w/v) SDS, 10% glycerol (w/v), 1 mM sodium EDTA, and 0.004% (w/v) bromphenol blue, and subjected to electrophoresis on a 4–16% gradient polyacrylamide gel (22).

Protein bands were diffusion blotted on to polyvinylidene difluoride membranes. After blotting, membranes were cut, blocked with 0.5% (w/v) bovine serum albumin in phosphate-buffered saline containing 0.05% (v/v) Tween 20 (blocking solution) for 1 h, and incubated (1 h) with LUM5B-7(D2) (1:500) or LUM5ACB-2(D1) (1:500) antisera or the alkaline phosphatase-conjugated swine anti-rabbit antibody (1:2000 in blocking solution), and membranes were then incubated (1 h) with a horseradish peroxidase-conjugated swine anti-rabbit antibody (1:2000 in blocking solution), and bands were visualized using the ECL Western detection kit.

**Enzyme-Linked Immunosorbent Assay**—Analysis with the LUM5B-2 antisera was performed according to Ref. 4. After appropriate dilution in 4 m guanidinium chloride, 10 mM sodium phosphate buffer, pH 7, samples were coated onto multwell assay plates (3912, Falcon) overnight at 4 °C (drying). After washing, plates were blocked (1 h) with blocking solution and incubated (1 h) with the LUM5B-2, LUM5B-5(Cys), and LUM5B-10(D3) antisera diluted 1:1000, the LUM5B-7(D2) and LUM5ACB-2(D1) antisera diluted 1:500, and the anti-albumin antibody diluted 1:300. Reactivity was detected with an alkaline phosphatase-conjugated swine anti-rabbit antisera, diluted 1:2000 in blocking solution using nitrophenyl phosphate (2 μg/ml in 1× diethanolamine buffer, pH 9.26) as a substrate. Reactivity is expressed as absorbance at 405 nm after 1 h.

**Immunohistochemistry**—Human submandibular gland tissue was fixed in 10% neutral buffered formalin overnight, dehydrated, and embedded in paraffin, and 4-μm sections were cut. Human tracheal tissue was fixed in ethanol and embedded in paraffin, and 4-μm sections were cut. Sections were dewaxed, rehydrated, and treated with 10 mM sodium citrate buffer, pH 6, at 100 °C for 10 min. Sections were then reduced using dithiothreitol in 0.1× Tris/ HCl buffer, pH 8.0, at room temperature (30 min) and alkylated with 25 mM iodoacetamide in 0.1× Tris/ HCl buffer, pH 8.0 (30 min). Endogenous peroxidase activity was quenched by immersion in 3% H2O2 for 30 min. Sections were placed in Coverplate immunostaining chambers (Shandon) and blocked with goat serum for 1 h before endogenous biotin was blocked (1 h) followed by incubation (1 h) with goat anti-human MUC5B-5(1-Cys) (diluted 1:1000 in blocking solution) followed by 1 h incubation with biotin hydrazide (1 h). After 20 min, the slides were washed with phosphate-buffered saline containing 0.05% (v/v) Tween 20 (washing solution). The slides were then subjected to peroxide oxidation, using 25 mM metaperiodate in 0.1× sodium acetate buffer, pH 5.5, for 20 min followed by incubation with biotin hydrazide (2.5 μg/ml in 0.1× sodium acetate buffer, pH 5.5) for 1 h. Alkaline phosphatase-conjugated streptavidin (1:20,000) in washing solution (containing 0.5% bovine serum albumin) was added (1 h), and bound reagent was detected using nitrophenyl phosphate as described for enzyme-linked immunosorbent assay above.

**RESULTS**

**Immunohistochemistry**—The antisera raised against the different N-terminal domains of MUC5B were investigated with immunohistochchemistry using tissues known to produce MUC5B, LUM5B-5(Cys), LUM5B-10(D3), LUM5ACB-2(D1), and LUM5B-7(D2) displayed strong reactivity over the mucous cells in the submucosal glands of human tracheal tissue (Fig. 1, a–d), as is true for the LUM5B-2 antisera (4). On submandibular gland tissue, the LUM5B-5(Cys) and LUM5B-10(D3) antisera reacted strongly (Fig. 1, e and f), whereas the LUM5ACB-2(D1) antibody was much weaker and the LUM5B-7(D2) antisera did not show any reactivity (results not shown). For some of the antisera, a weak reactivity was seen with the pre-immune sera over non-mucin-producing structures.
Density-gradient Centrifugation—HSMSL saliva was collected from three individuals (data shown for one) and separated into a sol and a gel phase. Following extraction of the gel with guanidinium chloride, the remaining pellet was brought into solution by reduction. Three fractions were thus obtained and are referred to as: “sol,” “gel-soluble,” and “gel-insoluble.” All samples were subjected to density-gradient centrifugation in CsCl.

In the sol phase, carbohydrate analysis revealed a population with a buoyant density between 1.40 and 1.32 g/ml reacting with both PAS and the glycan detection method (Fig. 2a). A major part of this material comprises the MUC7 mucin (14). No MUC5B reactivity was observed using either the LUM5B-2 or the LUM5B-5(1-Cys) antisera (Fig. 2b). The LUM5ACB-2(D1) and LUM5B-7(D2) antisera reacted with low density material at the top of the gradient, whereas the LUM5B-10(D3) antibody showed no reactivity (Fig. 2b). In the gel-soluble fraction, two major populations reacting with PAS (as well as with the glycan detection method) were detected at 1.45 and 1.40 g/ml, respectively (Fig. 2c), and reactivity with the LUM5B-2 and LUM5B-5(1-Cys) antisera followed the higher density one (Fig. 2d). In contrast, no reactivity was seen using the LUM5ACB-2(D1), LUM5B-7(D2), or LUM5B-10(D3) antisera (Fig. 2d). In the gel-insoluble fraction, both PAS and the glycan detection method revealed a population at 1.45 g/ml (Fig. 2c), and reactivity with the LUM5B-2, LUM5B-5(1-Cys), and LUM5B-10(D3) antisera coincided with the carbohydrate analyses (Fig. 2f). The LUM5ACB-2(D1) and LUM5B-7(D2) antisera showed no reactivity.

**DISCUSSION**

Antisera were raised against peptides within the D1, D2, D3, and the first Cys domain of MUC5B to investigate whether proteolytic cleavage within the N-terminal part of the apoprotein occurs in this mucin. The antisera were investigated using immunohistochemistry, and LUM5B-5(1-Cys), LUM5B-10(D3), LUM5ACB-2(D1), and LUM5B-7(D2) all reacted strongly over the mucous cells of the tracheal submucosal glands known to produce MUC5B (4). The LUM5B-5(1-Cys) and LUM5B-10(D3) antisera showed strong reactivity also on submandibular gland tissue sections, whereas the LUM5ACB-2(D1) and LUM5B-7(D2) antisera displayed weak or no reactivity. Possibly, the lack of reactivity of the two latter antisera in salivary tissue is explained by differences in macromolecular organization within the secretory granules reflected in the observation that a much larger proportion of salivary MUC5B occurs as an insoluble complex than respiratory MUC5B. Alternatively, the fact that the tissue from the salivary gland was fixed in formalin, whereas that from human trachea was fixed in ethanol,
could explain the differences. However, the results show that all antibodies do recognize the MUC5B apoprotein. Antisera against sequences within the D1 and D2 domains of MUC5B reacted with low density material at the top of the gradient and did not recognize the main mucin populations from the gel-soluble fraction and the gel-insoluble fraction. Consequently, a major part of the N-terminal end of MUC5B appears to be cleaved off, giving rise to a less glycosylated low density fragment that is separated from the highly glycosylated high density mucins. When the putative fragment was subjected to gel chromatography, the two antisera recognizing the D1 and D2 domain, respectively, identified the same peak, suggesting that they recognize the same component. No effects were observed after reduction, suggesting that the putative MUC5B fragment is a monomeric structure. SDS-PAGE/Western blotting of this component resulted in a well defined band, and again, the two antisera identified the same component, which is of approximately the same size as albumin. Because albumin is of approximately the same size as the combined D1 and D2 domains and because the antiserum against the D1 domain is raised against a sequence in the N-terminal part of this domain, we propose that the MUC5B fragment identified results from a cleavage event in the D1/H11032 domain (Fig. 6). The D1 and D2 domains of vWF (741 amino acids) comprise the so-
called vWF propolypeptide, which is crucial for the formation of the oligomeric form of this protein. Cleavage of the vWF propolypeptide occurs in the D/H11032 domain between Arg and Ser residues in positions Lys 762-Arg763-Ser764 (25). This KRS sequence is not present in the N-terminal end of MUC5B, but there are several other paired basic amino acid sites that may provide substrate (for example, the proprotein convertases known to cleave at such sites (for a review, see Ref. 26)). If indeed cleavage occurs in the D/H11032 domain, the Arg 843-Arg844 sequence could be a potential site. However, whether the cleavage events in MUC5B reported here influence assembly of the mucin is not clear at present.

In a previous study, we have shown that the major part of the MUC5B mucin from HSMSL saliva is insoluble, i.e. it resists extraction with 6 M guanidinium chloride (16). The structural basis for this is not known; however, the complex can be brought into solution by cleaving disulfide bonds. Using rate-zonal centrifugation, a second fragment was identified in the preparation of soluble MUC5B using an antibody recognizing a sequence in the D3 domain. This component is likely to be highly glycosylated since it banded with the main mucin population. The fragment is apparently only released from soluble MUC5B because the reactivity with the antiserum against the D3 domain is retained with the mucin subunits from insoluble MUC5B following rate zonal centrifugation. The significance of this cleavage event remains to be established; however, it represents the first structural difference identified between soluble and insoluble salivary MUC5B. The possibility that differences in the solubility/structure of mucins may be determined by proteolytic cleavage events provides a conceptually new idea on how the properties of mucus, and thus the protection of mucosal surfaces, may be regulated and modulated between tissues to meet local physiological demands.

Extensive studies on PSM have shown that this mucin forms C-to-C dimers in the endoplasmic reticulum (12, 13) followed by N-to-N multimerization through the D-domains in the distal part of the Golgi (10). CGLCG sequences in the D1 and D3 domains have been suggested to be crucial for N-to-N multimerization, and these sequences are highly conserved in other secreted mucins, including MUC5B. No evidence for proteolytic cleavage in the N-terminal part of PSM was found (10). Although the D1 domain in MUC5B from human saliva seems to be cleaved off and the soluble MUC5B species apparently lack the D3 domain, this glycoprotein is still an oligomeric structure. Consequently, the postulated N-to-N-terminal association in human salivary MUC5B oligomers must occur in the C-
terminal part of the D3 domain and/or in domains that are C-terminal of this structure (Fig. 6). Possibly, additional disulfide bonds within the D3 domain provide the cross-link(s) necessary to form insoluble MUC5B. In the prepro-vWF, non-covalent interactions between the D1 and D2 domains are thought to "align" the D3 and A domains, which then form disulfide bonds between the vWF dimers (25, 27). Analogously, the role of the D1 and D2 domains in MUC5B might be to act as "docking" structures that after "alignment" and disulfide bond formation may be removed without compromising the oligomeric mucin structure. Free vWF propolypeptide is found as dimers associated by non-covalent bonds in plasma (28) in keeping with this idea. The apparent difference in N-terminal cleavage between PSM and human salivary MUC5B could be due to species differences, a difference between individual mucins, or possibly a lack of the enzyme responsible for cleavage in the cells used to study the PSM constructs. Alternatively, the N terminus of MUC5B may be a post-secretory event mediated by salivary proteases co-secreted with HSMSL. N-terminal cleavage is not essential for vWF oligomerization (25), and oligomerization could well proceed without N-terminal cleavage. To our knowledge it is not known whether or not PSM isolated from gland tissue has been subjected to N-terminal cleavage. To our knowledge it is not known whether or not PSM isolated from gland tissue has been subjected to N-terminal cleavage.

In this study, we demonstrate that proteolytic cleavage may occur in the N terminus of MUC5B, giving rise to a fragment that corresponds in size to the vWF propeptide and thus adding further evidence to the postulated similarities between this protein and the human oligomeric mucins. A second cleavage event may provide a structural explanation for the difference between soluble and insoluble MUC5B in saliva.

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