Identification of the Half-cystine Residues in Porcine Submaxillary Mucin Critical for Multimerization through the D-domains

ROLES OF THE CGLCG MOTIF IN THE D1- AND D3-DOMAINS

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Juan Perez-Vilar and Robert L. Hill‡
From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Plasmids encoding the amino-terminal region of porcine submaxillary mucin were modified by site-specific mutagenesis to assess the roles of individual half-cystine residues in the assembly of disulfide-linked multimers of mucin. COS-7 cells with the plasmid containing C1199A expressed primarily monomers, suggesting that half-cystine 1199 in the D3-domain is involved in forming mucin multimers. This residue is in the sequence C1199SWRYEPCG, which is highly conserved in the D3-domain of other secreted mucins and human propro-von Willebrand factor. In contrast, cells with the plasmid containing C1276A expressed trimers like those with unmutated plasmid, suggesting that half-cystine 1276 is not involved in formation of disulfide-bonded multimers. The roles of the half-cystines in the CGLCG motifs in the assembly of disulfide-bonded multimers of mucin were also assessed. Cells with plasmids in which both half-cystines in the motif in the D1- or D3-domain of mucin are replaced by alanine expressed proteins that were poorly secreted, suggesting that these mutations impair normal folding of the expressed proteins. A plasmid with a mutant D1-domain motif expressed monomers, whereas one with a mutant D3-domain motif expressed monomers and trimers. However, the trimers expressed by the latter plasmid were assembled in non-acidic compartments, as judged by expression studies in the presence of monensin, which inhibits trimer formation by unmutated plasmid, but not by the mutant plasmid. These results suggest that the CGLCG motif in the D1-domain is required for multimerization in the trans-Golgi complex. However, the CGLCG motif in the D3-domain appears to prevent formation of mucin multimers in non-acidic compartments of the cell. Plasmids encoding the D1- and D2-domains, the D1- and D3-domains, or only the D3-domain also expressed oligomers in the presence of monensin, suggesting that the three D-domains must be contiguous to avoid multimerization in non-acidic compartments. It is possible that these motifs in mucins are engaged in the thiol-disulfide interchange reactions during the assembly of disulfide-bonded multimers of mucin.

SECRETORY MUCINS ARE SYNTHESIZED BY EPITHELIAL CELLS THAT LINE THE LUMINAL SURFACES OF THE RESPIRATORY, GASTROINTESTINAL, AND UROGENITAL TRACTS OF VERTEBRATES, WHERE THEY SERVE TO PROTECT THE CELLS FROM PHYSICAL INJURY, DEHYDRATION, AND MICROBIAL INFECTION. THEY ARE ALSO FOUND ON THE SKIN OF AMPHIBIA. ALL OF THESE GYCOPROTEINS HAVE IN COMMON A CENTRAL DOMAIN CONTAINING MULTIPLE, TANDEMLY REPEATED SEQUENCES RICH IN SERINE AND THREONINE TO WHICH O-LINKED OLIGOSACCHARIDES ARE ATTACHED. HOWEVER, THE NUMBER, LENGTH, AND AMINO ACID SEQUENCES OF THE REPEATS VARY AMONG DIFFERENT MUCINS (REVIEWED IN REF. 1). ONE GROUP OF SECRETED MUCINS, INCLUDING PORCINE SUBMAXILLARY MUCIN1 (2–4), FROG INTEGRUMENTARY MUCIN FIM-B.12 (5–7), HUMAN MUCIN MUC23 (8–10) AND ITS RAT HOMOLOGUE rMUC24 (11–14), AND HUMAN MUCIN MUC3AC5 (15–18), HAS ANALOGOUS HALF-CYSTINE-RICH DOMAINS AT THEIR AMINO AND CARBOXYL TERMINI. THE ROLES OF THESE DISULFIDE-RICH DOMAINS IN THE ASSEMBLY OF PORCINE SUBMAXILLARY MUCIN INTO MULTIMERS LINKED BY DISULFIDE BONDS HAVE BEEN REPORTED EARLIER (19–21). THE SIMILARITY IN THE DOMAIN STRUCTURES OF MANY OF THE SECRETED MUCINS SUGGESTS THAT THEY SHARE SIMILAR MECHANISMS OF ASSEMBLY.

Porcine submaxillary mucin contains multiple tandem repeats, 81 residues in length with identical amino acid sequences (4). The polypeptide backbone of submaxillary mucin is encoded by one polymorphic gene with at least three alleles, each encoding different numbers of repeats (90, 125, and 133, respectively) (4). The polypeptide contains up to 13,288 residues (4), with a disulfide-rich region at its amino terminus containing the D1-, D2-, and D3-domains first identified in human prepro-von Willebrand factor6 (22) and human mucin MUC2 (10), but now recognized to occur in many proteins, including other secreted mucins, Bombyx mori hemocytin7 (23), mouse α-tectorin8 (24), mouse9 (25) and porcine10 (26) zonadhesins, and mouse otogelin11 (27). A 240-residue half-cystine-rich domain is at the carboxyl terminus of porcine mucin, and the half-cystines in this domain are in positions corresponding to the half-cystines in carboxyl-terminal disulfide-rich domains of prepro-von Willebrand factor, hemocytin, otogelin, and the secreted mucins listed above.

Porcine submaxillary mucin is N-glycosylated and forms disulfide-linked dimers through its carboxyl-terminal disulfide-rich domains while in the endoplasmic reticulum (19, 21). Only the most carboxyl-terminal 90 residues in the mucin including 11 half-cystines are involved in dimer formation (20). The half-
cystines in the 90-residue carboxyl-terminal sequence are in positions corresponding to those in Norrie disease protein (norrin), a 133-residue protein that forms disulfide-linked dimers and oligomers (28). One or more of the half-cystine residues at positions 13223, 13244, and 13246 in the norrin-like domain are involved in dimer formation of submaxillary mucin (20). Mucin dimers are O-glycosylated in the Golgi complex (29, 30) and, upon reaching the trans-Golgi compartments, are assembled into disulfide-bonded multimers via disulfide-bonded trimers of the amino-terminal D-domains (21). Unlike human prepro-von Willebrand factor (31), there is no proteolysis of the mucin D-domains by furin prior to secretion (21). Mucin multimerization appears to occur at a slightly acidic pH maintained by vacuolar H^+ -ATPase in the trans-Golgi compartments (21). Although the covalent assembly of other mucins has not been established to any great extent, rat rMuc2 is reported to form disulfide-linked dimers through its carboxyl-terminal disulfide-rich domains (32), and human MUC2 synthesized by LS174T cells forms disulfide-linked dimers while in the endoplasmic reticulum (33, 34).

To obtain further insight into the half-cystines involved in forming disulfide-linked multimers, the proteins expressed by cells transfected with plasmids encoding half-cystine mutants of the amino-terminal region of porcine submaxillary mucin have been examined. This study has identified half-cystine 1199 and the two half-cystines in the CGLCG motif in the D1-domain to be involved in forming multimers and the two half-cystines in the CGLCG motif in the D3-domain to impede formation of multimers of mucin in non-acidic compartments.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Vectors**—The expression vector pSMD1D2H was prepared from pMNH (21), which encodes the entire amino-terminal region containing the D1-, D2-, and D3-domains of submaxillary mucin (residues 1–1360) plus a six-histidine tag at its carboxyl terminus. pMNH was digested with PstI and religated to create pMD1D2H. This vector encodes the complete D1- and D2-domains of mucin (residues 1–835) followed by mucin residues 1172–1276, and only those cystines in the CGLCG motif in the D3-domain to impede formation of multimers of mucin in non-acidic compartments.

**RESULTS**

**Trimer Formation and Secretion of the C1199A and C1276A Mutants of the Amino-terminal Region of Mucin**—Half-cystine 1142 (36) and half-cystine 1225 (37) have been suggested to form interchain disulfide bonds in multimers of human prepro-von Willebrand factor. The corresponding half-cystines in porcine submaxillary mucin are half-cystines 1199 and 1276, which are located in the amino-terminal D3-domain (Fig. 1). The codons for these half-cystines were changed one at a time by site-specific mutagenesis to alanine codons in plasmid pSMNH (21), which encodes the three D-domains of mucin plus a His tag (Fig. 1). The mutant plasmids (C1199A and C1276A) were then transiently expressed in COS-7 cells, and 48 h after transfection, the cells were incubated in medium containing [35S]cysteine. Radiolabeled proteins from the medium were then analyzed by reducing SDS-gel electrophoresis and autoradiography. As shown in Fig. 2, both mutant plasmids expressed a single protein with M_r = 200,000 on reducing SDS-gel electrophoresis (lanes 2 and 3). These proteins were indistinguishable from that expressed by unmutated plasmid (lane 1), which, as shown earlier (21), corresponds to reduced N-glycosylated monomers of the amino-terminal region of mucin based on its sensitivity to N-glycanase and tunicamycin. These results show that secretion and likely N-glycosylation of the mucin amino-terminal region were not altered by either mutation.

Fig. 2 also shows the protein species expressed by cells transfected with the mutant plasmids when analyzed on nonreducing gels. As shown earlier (21), unmutated plasmid pSMNH (lane 4) produced monomers (M_r = 180,000) and a second, slow migrating disulfide-linked oligomeric form of mucin (lane 5) still insoluble in reducing gel. On SDS-gel electrophoresis, this oligomeric form, also as shown earlier (21), has a M_r of ~520,000 and migrates slower than dimeric fibronectin (M_r = 450,000) and faster than unreduced thyroglobulin (M_r = 660,000), which is consistent with disulfide-linked trimers. Mutant C1199A (lane 5) expressed almost entirely monomers (M_r ~ 180,000) and only...
small amounts of species the size of trimers. In contrast, mutant C1276A (lane 6) expressed both monomers and trimers similar in size to those produced by unmutated plasmid pSMNH (lane 4). Since multimerization of submaxillary mucin occurs by dimerization of the carboxyl-terminal disulfide-rich domains and subsequent oligomerization through the amino-terminal D-domains (19, 21), these observations suggest that half-cystine 1199, but not half-cystine 1276, is involved in formation of disulfide-linked multimers through the D-domains of the mucin.

**Oligomerization and Secretion of Truncated Mutants of the Amino-terminal Region of Mucin**—To determine the role of the three D-domains in mucin multimerization, three different plasmids were prepared and expressed in COS-7 cells. As shown schematically in Fig. 1, plasmid pSMD1D2H is devoid of the D3-domain and encodes the D1- and D2-domains with a carboxyl-terminal His tag. Plasmid pSMD1D3H is lacking the D2-domain and encodes a fusion protein containing the complete D1- and D3-domains with a carboxyl-terminal His tag. Plasmid pSMD3H lacks the D1- and D2-domains and encodes the entire D3-domain with a carboxyl-terminal His tag. Each plasmid was transfected into COS-7 cells, and the proteins expressed were isolated from the medium and analyzed under

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**Fig. 1.** Schematic structures of the proteins encoded by the constructs employed in this study. The domain structure of the polypeptide backbone of porcine submaxillary mucin is given at the top. The domain structures of the plasmid constructs are given below. The UD domains are the unique sequence domains that flank the repeat domain. There are up to 133 tandem repeats, most of which are omitted in the drawing. CD is similar to the C-domains of human von Willebrand factor. ND refers to the carboxyl-terminal disulfide-rich domain similar to that in norrin. The numbers given indicate the amino acid residues separating the domains, the half-cystines mutated by site-specific mutagenesis, or the residues forming the CGLCG sequence motifs also studied here. H6 indicates the six-histidine tag, and M1-R34 refers to the mucin signal peptide (residues 1–34).

**Fig. 2.** Expression and secretion of mutants C1199A and C1276A of the amino-terminal region of mucin. COS-7 cells transfected with the expression vector pSMNH (lanes 1 and 4), C1199A (lanes 2 and 5), or C1276A (lanes 3 and 6) were metabolically labeled 48 h after transfection with [35S]cysteine for 4 h. Proteins from the medium were purified by absorption on TALON-IMAC beads and analyzed by SDSPAGE electrophoresis and autoradiography after reduction in 2-mercaptoethanol (lanes 1–3) or without reduction (lanes 4–6). M and T indicate the positions of monomers and trimers, respectively, in unreduced samples. Top is the interface between the stacking and running gels. The molecular weights (MW) of the standards are in thousands.
Half-cystines Critical for Mucin Multimerization

FIG. 3. Expression and secretion of truncated mutants of the amino-terminal region of mucin. COS-7 cells transfected with the vector pSMNH (lanes 1 and 5), pSMD1D2H (lanes 2 and 6), pSMD1D3H (lanes 3 and 7), or pSMD3H (lanes 4 and 8) were metabolically labeled with [35S]cysteine for 4 h, and the proteins were purified and analyzed by SDS-gel electrophoresis in 2-mercaptoethanol (lanes 1–4) or without prior reduction (lanes 5–8) as described in the legend to Fig. 2. M and T refer to the positions of monomers and trimers, respectively, in lane 8, the proteins expressed by pSMD3H on unreduced gels. Top indicates the interface between the stacking and the running gels, and the molecular weights (MW) are in thousands.

Reducing conditions as described for Fig. 2. Fig. 3 shows that pSMD1D2H (lane 2) and pSMD1D3H (lane 3) expressed $M_r$ ~ 180,000 and 170,000 proteins, respectively, that were poorly secreted into the medium. In contrast, plasmid pSMD3H (lane 4) expressed the D3-domain ($M_r$ ~ 90,000) in amounts similar to those expressed by plasmid pSMNH (lane 1), which encodes the entire amino-terminal region (Fig. 1).

Fig. 3 also shows that without prior reduction, plasmid pSMD1D2H (lane 6) secreted, into the medium, monomers in very small amounts and mainly disulfide-linked oligomers that ran slightly faster than high molecular weight aggregates seen at the interface between the stacking and running gels. Plasmid pSMD1D3H (lane 7) expressed disulfide-linked oligomers and aggregates, but not monomers. Plasmid pSMD3H (lane 8) expressed primarily a protein with a size ($M_r$ ~ 240,000) similar to that of myosin ($M_r$ ~ 220,000), consistent with the formation of disulfide-linked trimers along with significant amounts of monomers ($M_r$ ~ 80,000) and very small amounts of disulfide-linked aggregates of the D3-domain. As expected, unmutated plasmid pSMNH (lane 5) expressed monomers and trimers, although the latter barely entered the running gel that was intentionally made with a higher percentage of acrylamide than the gel shown in Fig. 2.

Earlier studies (21) showed that interchain disulfide bond formation by the D-domains occurs in acidic compartments of the Golgi complex because monensin and other compounds that inhibit these compartments inhibit interchain disulfide bond formation. Fig. 4 shows the effects of monensin on the proteins expressed by pSMNH and pSMD3H. Confirming earlier studies (21), plasmid pSMNH secreted monomers under reducing conditions (lane 1) and monomers and disulfide-linked trimers under nonreducing conditions (lane 5). However, as also expected, in the presence of monensin, only monomers were observed under reducing (lane 2) and nonreducing (lane 6) conditions. In contrast, formation of trimers of the D3-domain by plasmid pSMD3H was unaffected by monensin (lane 8), although no interfacial aggregates were observed. The D3-domain secreted in the presence of monensin migrated slightly faster than that in its absence (lane 7), suggesting that this domain is N-glycosylated and that processing of N-linked oligosaccharides is impaired just as for the entire amino-terminal region (21). On reducing gels, monomers of the D3-domain were observed in the presence (lane 4) and absence (lane 3) of monensin. In similar experiments, monensin was found to be without effect on the formation of disulfide-linked species expressed and secreted by pSMD1D2H and pSMD1D3H (data not shown). These studies suggest that proteins lacking the D2- or D3-domain or both the D1- and D2-domains likely form disulfide-linked oligomers in non-acidic compartments of the secretory pathway, i.e. the endoplasmic reticulum and/or the cis- and medial-Golgi compartments (e.g. Ref. 38). Moreover, these studies also suggest that mucin D-domains have an inherent tendency to form interchain disulfide bonds at neutral pH, i.e. soon after their synthesis in the endoplasmic reticulum, but this is prevented when the D1-, D2-, and D3-domains are part of the same polypeptide chain.

Expression of Mutants C253A/C256A and C1058A/C1061A of the Amino-terminal Region of Mucin—Submaxillary mucin has two CGLCG amino acid sequence motifs, one in the D1-domain (residues 253–257) and another in the D3-domain (residues 1058–1062) (Fig. 1) (4), that are conserved in the corresponding D-domains of other mucins and human prepro-von Willebrand factor. To obtain insight into the possible role of this motif in formation of disulfide-linked multimers of mucin, each half-cystine in the two CGLCG motifs of submaxillary mucin was changed to alanine by site-directed mutagenesis of pSMNH (21), which encodes all three D-domains and a His tag. Mutant C253A/C256A (Fig. 1) has the CGLCG motif only in the D3-domain, and mutant C1058A/C1061A (Fig. 1) has the motif only in the D1-domain. Pulse-chase studies were performed to investigate the fate of the mutant proteins expressed in COS-7 cells. Fig. 5A shows the proteins synthesized and secreted into the medium by each mutant compared with those synthesized and secreted by unaltered pSMNH. At the end of the pulse with [35S]cysteine in the absence (lanes 1, 3, 5, and 7) and presence (lanes 2, 4, 6, and 8) of 10 μg/ml monensin. The proteins were purified from the medium and analyzed by reducing (lanes 1–4) or nonreducing (lanes 5–8) SDS-gel electrophoresis and autoradiography as described in the legend to Fig. 2. M and T indicate the positions of monomers and trimers, respectively, in lanes 7 and 8, the proteins expressed by pSMD3H on unreduced gels. Top indicates the interface between the stacking and running gels, and the molecular weights (MW) are in thousands.

In summary, these studies indicate that the CGLCG motif functions to direct the D-domains into disulfide-linked multimers, presumably by directing the D-domains into interchain disulfide bonds. The D-domains of the D1-domain and D3-domain are required for multimerization. The D2-domain is not required for multimerization, but it may facilitate multimerization by promoting the formation of disulfide-linked trimers.
that were secreted into the medium by cells containing mutant C253A/C256A or C1058A/C1061A. For these studies, a larger number of transfected cells were used, and the proteins were isolated by immunoprecipitation with antisera 5287, solubilization of the precipitates in buffered 6 M guanidine HCl, and absorption of the proteins on TALON-IMAC beads. This method provided enough protein to observe on gels and reduced the background proteins associated with the larger number of cells. Mutant C253A/C256A (lane 6) expressed almost entirely monomers and very small amounts of disulfide-linked trimers, but both species migrated at the same positions as the proteins secreted from cells containing unmutated pSMNH (lane 3). In contrast, cells expressing mutant C1058A/C1061A (lane 9) secreted substantial amounts of both monomers and disulfide-linked trimers along with disulfide-linked aggregates at the stacking/running gel interface. These results suggest that the CGLCG motif in the D1-domain is required for efficient trimerization of the amino-terminal region in the trans-Golgi compartments.

The proteins secreted into the medium by cells containing mutant C1058A/C1061A were examined as described for Fig. 5B, except that the cells were also grown in the presence of monensin, and only the proteins in the medium were examined. Fig. 6 shows that the proteins secreted in the absence (lane 5) or presence (lane 6) of monensin on nonreducing gels were a mixture of monomers, trimers, and aggregates. The monomers in these lanes migrated as a mixture of two broad bands, which were shown earlier for the proteins expressed by unmutated pSMNH to be glycosylated and unglycosylated monomers (21). As controls, lanes 1 and 4 show the proteins expressed by mutant C253A/C256A in the absence of monensin. The glycosylated species expressed in the presence of monensin migrated slightly faster than those secreted in its absence. On reducing SDS gels, only glycosylated and unglycosylated monomers were observed for both mutants (lanes 2 and 3), although the glyco- and monensin monomers secreted in the presence of monensin migrated faster than those secreted in its absence, as noted earlier (21). Thus, monensin did not inhibit trimerization or aggregation of mutant protein. This result suggests that trimer formation of a mucin amino-terminal region with a mutated CGLCG motif in the D3-domain can occur at neutral pH, i.e.

**Fig. 5.** Pulse-chase studies of the expression of mutants C253A/C256A and C1058A/C1061A in COS-7 cells. COS-7 cells were transfected with plasmid pSMNH (lanes 1–3), C253A/C256A (lanes 4–6), or C1058A/C1061A (lanes 7–9) and, 48 h after transfection, metabolically labeled with [35S]cysteine for 15 min (lanes 1, 4, and 7) and then chased for 2 h in medium containing an excess of unlabeled cysteine (lanes 2, 3, 5, 6, 8, and 9). Proteins from detergent extracts of cells (C; lanes 1, 2, 4, 5, 7, and 8) or from the medium (M; lanes 3, 6, and 9) were immunoprecipitated with antiserum 5287 and analyzed by SDS-gel electrophoresis and autoradiography. Ten times the amount of medium from cells transfected with the mutant plasmids (lanes 4–6) was used for purification compared with the amount of medium used from cells transfected with pSMNH, which were further purified by absorption on TALON-IMAC beads. M and T indicate the positions of monomers and trimers, respectively, in lanes 2, 3, 6, and 9 in B. Top indicates the interface between the stacking and running gels, and the molecular weights (MW) are in thousands. A, electrophoresis in 2-mercaptoethanol; B, electrophoresis under nonreducing conditions.

**Fig. 6.** Effect of monensin on the secretion and trimerization of mutant C1058A/C1061A expressed in COS-7 cells. COS-7 cells transfected with the plasmid containing C253A/C256A (lanes 1 and 4) or C1058A/C1061A (lanes 2, 3, 5, and 6) were incubated with [35S]cysteine for 15 min and then chased in unlabeled medium for 2 h in the absence (lanes 1, 2, 4, and 5) or presence (lanes 3 and 6) of monensin (10 μg/ml). Proteins from the culture medium were purified by immunoprecipitation and by absorption on TALON-IMAC beads and analyzed under reducing (lanes 1–3) or nonreducing (lanes 4–6) conditions by SDS-gel electrophoresis and autoradiography. M and T indicate the positions of monomers and trimers, respectively, in lanes 4–6. Top indicates the interface between the stacking and running gels, and the molecular weights (MW) are in thousands.
Half-cystines Critical for Mucin Multimerization

The studies described here show that half-cystine 1199 in the D3-domain of porcine submaxillary mucin (PSM) (4) with the corresponding sequences in frog integumentary mucin FIM-B.1 (7), rat mucin rMuc2 (14), human mucins MUC5AC (18) and MUC2 (10), and human prepro-von Willebrand factor (pp-vWF) (40). The half-cystine residues that may be involved in multimerization of prepro-von Willebrand factor (half-cystines 1142, 1222, 1225, and C1227) are underlined. Also underlined are the half-cystine residues (positions 1199 and 1276) in porcine submaxillary mucin that were changed to alanine. Amino acids conserved among secretory mucins or among secretory mucins and prepro-von Willebrand factor are in boldface. B, alignment of amino acid sequences of porcine submaxillary mucin that contain the CGLCG motif in the D1- and D3-domains with the corresponding sequences in the active sites of Escherichia coli thiol-disulfide interchange protein (DSBC), Leishmania thioredoxin, and human protein-disulfide isomerase (PDI) (41) and also in the D1- and D3-domains of FIM-B.1, rMuc2, MUC2, MUC5AC, and prepro-von Willebrand factor. The half-cystine residues in submaxillary mucin that were changed to alanine by site-specific mutagenesis are underlined. The amino acid residues that are identical in the oxidoreductases and the D1- and D3-domains are indicated in boldface.

Fig. 4 showing that the D3-domain alone forms trimers, these species are likely to represent dimers linked through the carboxyl-terminal disulfide-rich domain that have been converted to multimers through the D3-domain. At the end of the 2-h chase, the higher molecular weight species increased in amount, whereas the monomers were not observed (lane 5). Very small amounts of only two proteins were secreted into the medium (lane 6). One is thought to be dimers of the species seen in lane 3 combined through disulfide bonds in the carboxyl-terminal disulfide-rich domain. The other is likely high molecular weight multimers of the dimers linked by disulfide bonds in the D3-domain. These results suggest that mucin multimerization in non-acidic compartments leads to a reduced rate of mucin secretion.

**DISCUSSION**

The studies described here show that half-cystine 1199 in the D3-domain of porcine submaxillary mucin forms an interchain disulfide bond in mucin multimers (Fig. 2). The corresponding half-cystine in human prepro-von Willebrand factor (half-cystine 1142) also forms an interchain disulfide bond (36), which indicates that the mechanism of multimerization of these proteins may be very similar, as suggested earlier (21). Half-cystine 1199 in mucin...
secretory mucins are indicated in boldface. The residues that are conserved in other half-cystines must also be involved. This study, but other half-cystines must also be involved. The residues that are conserved in other secretory mucins are indicated in boldface.

... occurs in the sequence C^{1399}SWRYEPCG, in which six of the nine residues are identical to the corresponding residues (Fig. 8A) in the D3-domains of frong mucin FIM-B.1 (7), rat rMuc2 (14), and human mucins MUC2 (10) and MUC5AC (18). This sequence identity suggests that the corresponding half-cystines in the other mucins are also involved in forming interchain disulfide bonds in mucin multimers. However, at least one other half-cystine forms an interchain disulfide bond in submaxillary mucin multimers because the proteins expressed by the plasmid containing C1199A (Fig. 2, lane 5) include small amounts of trimers. This is not surprising since the trimeric model of mucin assembly (21) requires more than one disulfide bond. In contrast, mucin half-cystine 1276, which corresponds to another half-cystine in human prepro-von Willebrand factor (half-cystine 1225) that has been suggested to form interchain disulfide bonds (37), does not appear to form interchain disulfide bonds in submaxillary mucin because the plasmid containing C1276A expresses trimers (Fig. 2, lane 6). These trimers have a slightly higher molecular weight than those expressed by unmutated plasmid, but this is believed to result from disruption of an intrachain disulfide bond formed by half-cystine 1276. The results with the mucin mutant plasmid containing C1276A are consistent with the observation that constructs encoding the entire polypeptide of prepro-von Willebrand factor, except with half-cystine 1225 replaced by glycine or alanine, form the same multimers as the unmutated construct (36). Moreover, the lack of highly significant sequence identities between CGLCG motifs of porcine submaxillary mucin result in proteins expressed by the plasmid containing C1199A (Fig. 8A) argues further against half-cystine 1276 forming interchain disulfide bonds in mucin multimers.

The locations of the CGLCG motifs are conserved in the amino-terminal D1-and D3-domains of many secreted mucins, human prepro-von Willebrand factor (14), mouse α-tectorin (24), and mouse (25) and porcine (26) zonadhesins. However, prepro-von Willebrand factor, α-tectorin, the zonadhesins, and human MUC5AC (18) have an additional CGLCG motif in their D2-domains, whereas porcine submaxillary mucin (4), frog FIM-B.1 (7), rat rMuc2 (14), and human MUC2 (10) have the sequence GLCG. Mutations of the half-cystines in either of the CGLCG motifs of porcine submaxillary mucin result in proteins that are poorly secreted (Fig. 5A), suggesting that the folding of the mutant proteins is impaired. Possibly a disulfide bond critical to correct folding is disrupted, or the mechanism of folding is indirectly affected. However, we have shown here that plasmids lacking the D2- or D3-domain express proteins that are poorly secreted (Fig. 3), suggesting that somehow the different D-domains must interact with one another for correct folding. Because the CGLCG motif resembles the active-site motifs (CGXC) in proteins catalyzing disulfide bond formation during protein folding, including protein-disulfide isomerase, thioredoxin, and thiol-disulfide interchange protein (reviewed in Ref. 41) (Fig. 8B), it is possible that the mucin D-domains interact with one another and with mucin half-cystines in the thiol-disulfide interchange reactions, leading to correct folding and disulfide bond formation.

The first step in assembly of mucin into disulfide-linked multimers is the formation of mucin dimers linked by disulfide bonds in the carboxyl-terminal norrin-like domain (19, 20). This step occurs in the endoplasmic reticulum, where N-glycosylation also takes place. After O-glycosylation in the cis- and medial-Golgi compartments (29, 30), mucin dimers then form multimers by disulfide bonding among D-domains in the acidic compartments of the trans-Golgi complex (21). Multimer formation does not occur in cells treated with compounds that increase the pH of the acidic compartments or that inhibit the vacuolar H⁺-ATPase involved in acidification of the compartments (21). A plasmid with a mutant CGLCG motif (AGLAG) in the D3-domain can make trimers; however, this occurred in non-acidic compartments as judged by the failure of monensin to inhibit the process (Fig. 6). These results suggest that the CGLCG motif in the D3-domain prevents formation of interdimeric disulfide bonds in non-acidic compartments, i.e. the endoplasmic reticulum and the cis- and medial-compartments of the Golgi complex (38). Such a mechanism gains support from the observation that plasmids expressing the D1- and D2-domains, the D1- and D3-domains, or only the D3-domain express disulfide-linked oligomers assembled in non-acidic compartments (Fig. 4). Thus, the D-domains have an inherent tendency to form interchain disulfide bonds in non-acidic compartments, which is inhibited when all three D-domains are contiguous. Moreover, mucin multimers formed in non-acidic compartments are very likely poorly secreted from the cells, as judged by studies with a plasmid encoding only the D3-domain and the entire 240-residue carboxyl-terminal disulfide-rich domain (Fig. 7). Therefore, it appears that interdimeric disulfide bond formation in the endoplasmic reticulum or the cis- and medial-Golgi compartments is detrimental and must be avoided. The way that the CGLCG motif in the D3-domain accomplishes this is unclear. Nevertheless, preliminary studies suggest that a mutation in the CGLCG motif in the D3-domain cannot be complemented by coexpression in the same cell of a mucin domain with an intact motif, suggesting that its proposed function depends upon precise interactions among D-

12 GenBank access number J05016.
13 GenBank access number AE001274.
14 GenBank access number U32800.
domains in each mucin molecule. Indeed, coexpression in the same cells of plasmids encoding the mucin amino-terminal region and small (14–20 residues) polypeptides containing either of the CGLCG motifs does not alter the rate of secretion or trimerization of the mucin region (data not shown).

Mutant plasmids with the CGLCG motif in the D1-domain express primarily mucin monomers (Fig. 5B). The results given in Fig. 2 show that half-cystine 1199 in the D3-domain is critical for formation of disulfide-bonded multimers, but secretion of monomers is unimpaired. This observation and the fact that the D3-domain of prepro-von Willebrand factor (42) since alteration of either motif prevented multimerization of the factor.

The motif GLCG in the D2-domain of submaxillary mucin may contribute to the proposed functions of the CGLCG motifs. In the oxidoreductases, in porcine submaxillary mucin in the sequence that connects the D1- and D2-domains. This motif, C^{905}NKC, is conserved in the corresponding regions of FIM-B1 (C^{948}DKC), MUC2 (C^{839}NTC), and MUC5AC (C^{882}NTC) and may contribute to the proposed functions of the CGLCG motifs. The motif GLCG in the D2-domain of submaxillary mucin may also play an important role since it is conserved in frog FIM-B1, rat MUC2, and human MUC2 and is the only motif of this kind found in the D-domains of B. mori hemocytin and mouse otogelin.

Fig. 9 summarizes schematically the half-cystines identified in the present and previous studies (20, 21) that are thought to be involved in the processing and assembly of porcine submaxillary mucin. Interestingly, all of these half-cystines are in amino acid sequences that are highly conserved in many secretory mucins; thus, it seems very likely that the corresponding half-cystines in other secretory mucins play similar roles.

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Juan Perez-Vilar and Robert L. Hill

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