Porcine Submaxillary Mucin Forms Disulfide-linked Multimers through Its Amino-terminal D-domains*

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COS-7 cells expressing 1,360 residues from the amino terminus of porcine submaxillary mucin were used to determine whether this region, containing the D1, D2, and D3 domains, is involved in forming mucin multimers. Analysis of the proteins immunoprecipitated from the medium of transfected cells by reducing SDS-gel electrophoresis showed a single N-glycosylated protein with no indication of proteolytically processed forms. Without prior reduction, only two proteins, corresponding to monomeric and disulfide-linked trimeric species, were observed. The expressed protein devoid of N-linked oligosaccharides also formed trimers, but was secreted from cells in significantly less amounts than glycosylated trimers. Pulse-chase studies showed that the disulfide-linked trimers were assembled inside the cells no earlier than 30 min after protein synthesis commenced and after the intracellular precursors were N-glycosylated. Trimer formation was inhibited in cells treated with brefeldin A, monensin, chloroquine, or bafilomycin A1, although only brefeldin A prevented the secretion of the protein. These results suggest that trimerization takes place in compartments of the Golgi complex in which the vacuolar H+-ATPase maintains an acidic pH. Coexpression in the same cells of the amino-terminal region and the disulfide-rich carboxyl-terminal domain of the mucin showed that these structures were not disulfide-linked with one another. Cells expressing a DNA construct encoding a fusion protein between the amino- and carboxyl-terminal regions of the mucin secreted disulfide-linked dimeric and high molecular weight multimeric species of the recombinant mucin. The presence of monensin in the medium was without effect on dimerization, but inhibited the formation of disulfide-linked multimers. These studies suggest that disulfide-linked dimers of mucin are subsequently assembled into disulfide-linked multimers by the amino-terminal regions. They also suggest that the porcine mucin forms branched disulfide-linked multimers. This ability of the amino-terminal region of mucin to aid in the assembly of multimers is consistent with its amino acid identities to the amino-terminal region of human von Willebrand factor, which also serves to form disulfide-linked multimers of this protein.

Porcine submaxillary mucin (GenBank™ accession number AF005273) contains up to 13,288 amino acid residues arranged in different domains characteristic of secretory mucins (1). A major central domain contains 90–135 81-residue tandem repeats rich in threonine, serine, glycine, and alanine, with the exact number of repeats depending on the mucin gene expressed (1). This central domain is flanked, at both ends, by unique domains with amino acid compositions similar to the repeat domain, but different in sequence from the repeat domain and one another. All of the threonine and serine residues in the tandem repeat and the unique domains appear to contain O-linked oligosaccharides (2). Flanking the unique sequence domains are three half-cystine-rich domains at the amino-terminal side and one (240 residues) disulfide-rich domain at the carboxyl-terminal side (1). The half-cystine residues present at the carboxyl-terminal end of submaxillary mucin are conserved in the corresponding regions of human von Willebrand factor (3) and many secretory mucins, including frog integumentary mucin FIMB1 (4); rat intestinal mucin (5); bovine submaxillary mucin (6); and human mucins MUC2 (7), MUC5B (8, 9), MUC5AC (10), and MUC6 (11). Moreover, the 11 carboxyl-terminal half-cystine residues in human von Willebrand factor and those in the above secretory mucins are homologous to the 11 half-cystine residues in Norrie disease protein (norrin) (12). It has been found that human von Willebrand factor (13) and pig submaxillary mucin (14) form disulfide-linked dimers through their respective carboxyl-terminal domains, whereas norrin forms disulfide-linked oligomers (15). The 1,350 amino acid residues in the amino-terminal region of submaxillary mucin are organized into three disulfide-rich homologous domains (1), named D1, D2, and D3, which share significant amino acid identities with the D-domains present in the amino-terminal regions of human prepro-von Willebrand factor (GenBank™ accession number X04385) (3), frog integumentary mucin FIMB1 (GenBank™ accession number Y08296) (16), and human mucin MUC2 (GenBank™ accession number L21998) (17). The D-domains in human pro-von Willebrand factor mediate the formation of disulfide-linked multimers of the protein (18), but the role of the corresponding D-domains in secretory mucins remains unknown.

We report here expression studies in mammalian cells of plasmids encoding the amino-terminal region of porcine submaxillary mucin. The mucin region is N-glycosylated and forms disulfide-linked trimers prior to secretion from the cells. Trimer formation is not dependent on N-linked oligosaccharides and takes place in acidic compartments of the Golgi complex. Expression studies of a construct encoding the mucin amino-terminal region and the entire mucin disulfide-rich carboxyl-terminal domain showed the formation of high molecular weight disulfide-bonded multimeric species.

EXPERIMENTAL PROCEDURES

Antisera against the Mucin Domains—Production of antiserum 3814 against the disulfide-rich carboxyl-terminal domain of mucin was described earlier (14). The rabbit antisera specific for the amino-terminal region of submaxillary mucin were obtained against mucin polypeptides
expressed in and purified from *Escherichia coli* by a glutathione S-transferase fusion expression system. For preparation of antisera, cDNA 4674, a cDNA fragment encoding amino acid residues 266–1014 of the salivary mucin (1) was amplified by polymerase chain reaction using *Elongase* (Life Technologies Inc.), a cDNA encoding the entire amino-terminal region of the mucin (1) as template, and the following primers: 5′-GAATTCCGGGTGAGTTGACACTCCTTACATTG-3′ and 5′-CTCGAGTTCGAAAAAGCCGCCGAGTTACATCCCTTACTTG-3′. The resulting fragment was subcloned into a pGEX-4T vector (Amersham Pharmacia Biotech) in frame with sequences encoding the glutathione S-transferase. To obtain antisera 5287, a mucin cDNA (encoding amino acid residues 1059–1360) was similarly amplified by polymerase chain reaction using the primers 5′-GAATTCCGGGTGAGTTGACACTCCTTACATTG-3′ and 5′-CTCGAGTTCGAAAAAGCCGCCGAGTTACATCCCTTACATTG-3′, and the resulting DNA fragment was subcloned into pGEX-4T. Both plasmids were expressed in *E. coli*, and the fusion proteins were purified by affinity chromatography on glutathione-agarose following published procedures (19, 20). The purified proteins were injected into rabbits (21) as fusion proteins (antisera 5287) or after removing the glutathione S-transferase sequences with thrombin (antisera 4674) (20).

**Construction of Mammalian Expression Vectors**—A DNA fragment encoding six consecutive histidine codons was inserted at the 3′-end of a cDNA encoding the entire amino-terminal region of submaxillary mucin (amino acid residues 1–1360) cloned into the pFastBac vector (1). The poly-His encoding fragment was made by annealing the following complementary oligonucleotides: 5′-GGCGGACCATCACCATCACCATCCTAGC-3′ and 5′-TGAAGCTTGAAGCTTGAAGCTTGAAGCTTGAAGCTTGAAGCTTGAAGCTT-3′. The mucin cDNA fragments, with or without the His tag coding sequence, were obtained as SacI/XhoI fragments and subcloned into the *XhoI* site of the pSMN plasmid. The pSMN expression vector, which encodes the amino-terminal region of porcine submaxillary mucin (residues 1–1360), was similarly amplified by polymerase chain reaction using the primers 5′-GAATTCCGGGTGAGTTGACACTCCTTACATTG-3′ and 5′-CTCGAGTTCGAAAAAGCCGCCGAGTTACATCCCTTACATTG-3′, and the resulting DNA fragment was subcloned into pGEX-4T. The purified proteins were reduced with 2-mercaptoethanol, His tag inserted at the carboxyl terminus of the mucin region.

**RESULTS**

**Expression, N-Glycosylation, and secretion of the amino-terminal region of mucin**—The pSMNH expression vector, which encodes the entire amino-terminal region of submaxillary mucin (residues 1–1360) plus a histidine tag (six residues) introduced at its carboxyl terminus, was expressed in COS-7 cells. Forty-eight hours after transfection, the cells were incubated in medium containing [35S]cysteine for 4 h. Proteins from the medium were immunoprecipitated with preimmune serum (lanes 1 and 3), antisera 4674 (lane 2), or antisera 5287 (lane 4) or absorbed to TALON-IMAC beads (lane 5) and analyzed by SDS-gel electrophoresis in 2-mercaptoethanol and autoradiography. The molecular weights of the standards are in thousands. *B*, COS-7 cells expressing the pSMNH vector were incubated in [35S]cysteine-containing medium for 4 h in the absence (lane 1) or presence (lane 2) of 10 μg/ml of tunicamycin, and the proteins were purified by absorption to and elution from TALON-IMAC beads and analyzed as described for *A*. C, 35S-labeled proteins secreted into the medium by COS-7 cells transfected with vector pSMNH were purified with TALON-IMAC beads, denatured by boiling in 2-mercaptoethanol in buffered SDS, and incubated with buffer alone (lane 1) or N-glycanase (lane 2) as reported earlier (14). The digests were analyzed as described for *A*.

**Expression, N-Glycosylation, and secretion of the amino-terminal region of mucin**—The pSMNH expression vector, which encodes the entire amino-terminal region of submaxillary mucin (residues 1–1360) plus a histidine tag (six residues) introduced at its carboxyl terminus, was expressed in COS-7 cells. Forty-eight hours after transfection, the cells were incubated in medium containing [35S]cysteine in the presence or absence of tunicamycin. Proteins were purified from the culture medium by immunoprecipitation with antisera 4674 prepared against amino acid residues 266–1014 of the mucin; with antisera 5287 prepared against residues 1059–1360 of the mucin; or with TALON-IMAC beads, which bind the poly-His tag inserted at the carboxyl terminus of the mucin region. The purified proteins were reduced with 2-mercaptoethanol and analyzed by SDS-gel electrophoresis and autoradiography. As shown in Fig. 1A, irrespective of the absorbent used, the amino-terminal region of mucin was secreted as a single protein with *Mr* ~ 200,000. When the addition of N-linked oligosaccharides was inhibited with tunicamycin, the protein migrated on reducing SDS gels with *Mr* ~ 165,000 (Fig. 1B), consistent with the observed size of proteins obtained by *in vitro* transcription/translation studies (data not shown). Digestion of the protein purified from the medium of untreated cells with N-glycanase resulted in a reduction in size similar to that found with protein expressed in the presence of tunicamycin.
(Fig. 1C). These results show that the amino-terminal region of mucin, containing the D1, D2, and D3 domains, is secreted as a single protein, which is N-glycosylated at several Asn-X-(Thr/ Ser) acceptor sites found in its sequence (1). They also show that N-glycosylation is not required for secretion of the mucin region, although the amounts of the protein recovered from the medium of cells incubated in tunicamycin are significantly lower than those secreted by untreated cells.

**Trimer Formation of the Amino-terminal Region of Mucin**—To determine whether the amino-terminal region of mucin forms disulfide-linked oligomers, the proteins expressed and secreted by pSMNH-transfected COS-7 cells incubated in the presence of [35S]cysteine were purified by immunoprecipitation with antisera 5287 or absorption to TALON-IMAC beads and analyzed by SDS-gel electrophoresis without prior reduction (Fig. 2A). Irrespective of the absorbents employed, two proteins were observed. One corresponded to a protein with a molecular weight of 165,000, which is indistinguishable from the mu-Scin—Scin formed in the presence of TALON-IMAC beads and analyzed by SDS-gel electrophoresis with and without reduction (Fig. 2A). These results show that the amino-terminal region of mucin is secreted as monomeric and disulfide-linked trimeric species. Fig. 3A shows that trimer formation was not inhibited by treatment of the cells with tunicamycin, indicating that N-glycosylation of the protein is not required for the formation of disulfide-linked trimers.

Secretion and trimer formation of the mucin amino-terminal region were also found to occur in a different cell line. Fig. 2D shows the electrophoretic pattern of proteins purified by immunoprecipitation with antisera 5287 from the medium of mouse MOP-8 cells transfected with plasmid pDMNH. Only monomers and high molecular weight trimers of the mucin region, identical to those protein species secreted by COS-7 cells, were observed. These results suggest that secretion and trimer formation of the amino-terminal region of mucin do not depend on the cell type.

**Rate of Trimer Formation and Secretion of the Amino-terminal Region of Mucin**—Pulse-chase studies with COS-7 cells transfected with the pSMN vector were performed to measure the rates of formation and secretion of the trimers formed by the amino-terminal region. After incubation (15 min) in medium containing [35S]cysteine, the cells were incubated in complete medium with an excess of unlabeled cysteine. At selected time periods, the cells were lysed with buffered detergents, and proteins were purified by immunoprecipitation with antisera 5287, reduced with mercaptoethanol, and analyzed by SDS-gel electrophoresis and autoradiography. As shown in Fig. 3A, the first intracellular protein detected had a molecular weight of 165,000 (lane 1), which is indistinguishable from the mucin expressed and purified from tunicamycin-treated cells (Fig. 1B) or after digestion of the expressed mucin with N-glycanase (Fig. 1C). This molecular species is thought to be protein synthesized in the endoplasmic reticulum but not yet glycosylated. A second intracellular, less abundant protein with a molecular weight of 200,000 appeared after 30 min of incubation in unlabeled medium (lane 3), just at the time when the amount of the initial unglycosylated precursor started to diminish, and reached a maximal amount at 60 min of chase (lane 4). This species is thought to be N-glycosylated mucin. At longer chase times, both the unglycosylated and glycosylated proteins disappeared from the cells. These observations indicate that N-glycosylation is a post-translational modification of the amino-terminal region of mucin. Fig. 3A also shows the proteins

![Fig. 2. Trimer formation of the amino-terminal region of mucin.](image-url)
analyzed on gels without prior reduction. A protein with $M_r$ were metabolically labeled with $[35S]$cysteine for 15 min and chased for COS-7 cells were transfected with plasmid pSMN, and 48 h later, cells the same amounts in all the time periods analyzed, including the pulse with $[35S]$cysteine ($150,000$ was the only species found in cell lysates at the end of the pulse ($lane 10$), at which time $N$-glycosylated monomers purif...
Evidence for the involvement of specific Golgi complex compartments in trimer formation of the mucin was obtained by incubating COS-7 cells expressing the mucin amino-terminal region with agents that disrupt the pH gradient of this organelle (reviewed in Ref. 23). As shown in Fig. 5A, incubation of the cells in the presence of the ionophore monensin (lane 2) or the lysosomotropic agent chloroquine (lane 3) did not abrogate the secretion of the mucin region, although the latter significantly reduced the amount of the protein in the medium. However, in both cases, the secreted proteins exhibited a Mr (175,000) on reducing SDS gels slightly lower than those of protein species purified from the medium of untreated cells (lane 1), suggesting that the processing of the N-linked oligosaccharides has been altered by the above compounds (23). Without prior reduction in 2-mercaptoethanol, only a single species corresponding to a protein of Mr ~170,000 was observed on SDS gels of proteins purified from cells incubated in the presence of monensin (lane 5) or chloroquine (lane 6), whereas untreated cells (lane 4) expressed and secreted both monomeric and trimeric species. These results indicate that monensin and chloroquine inhibited the formation of disulfide-linked trimers of the mucin region, suggesting that trimer formation requires a low pH. This possibility was further confirmed by incubating pSMNH-transfected COS-7 cells in medium containing bafilomycin A1 (24), a specific inhibitor of the vacuolar H\(^+\)-ATPase (reviewed in Ref. 25) assumed to maintain a pH between 5.9 and 6.6 in the trans Golgi compartments (e.g. Refs. 26–28). Fig. 5B shows that on reducing SDS gels, the species secreted in the presence of bafilomycin A1 (lane 2) presented a slightly lower Mr than those secreted by untreated cells (lane 1) and were indistinguishable from those secreted by cells incubated in the presence of monensin or chloroquine (Fig. 5A). Moreover, bafilomycin A1 also inhibited the formation of disulfide-linked trimers of the mucin region (lane 4). These results indicate that trimer formation requires the continuous activity of the vacuolar H\(^+\)-ATPase and suggest that trimer formation occurs in acidic compartments of the Golgi complex, which includes the trans cisternae and the trans tubular network (29).

Role of the Amino- and Carboxyl-terminal Domains in the Structure of Mucin—Previous studies (14) have shown that the carboxyl-terminal domain of submaxillary mucin forms disulfide-linked dimers between monomers. Therefore, it was important to determine whether the amino- and carboxyl-terminal regions of the submaxillary mucin form interchain disulfide bonds between one another. For this purpose, COS-7 cells were simultaneously transfected with pSMNH, which encodes the amino-terminal region of submaxillary mucin with six histidine residues at its carboxyl terminus, and pMC (14), a vector encoding the entire disulfide-rich carboxyl-terminal domain of the mucin. After incubation of the cells in medium with \(^{35}\)S-cysteine, the proteins secreted into the medium were immunoprecipitated with antiserum 3814, which recognizes the carboxyl-terminal domains (14) and trimerization among the amni-
no-terminal regions occur independently and account for the permanent interchain disulfide bonds formed during submaxillary mucin biosynthesis.

Pulse-chase studies with COS-7 cells transfected with the pSMNCH vector were performed to assess the formation of high molecular weight disulfide-linked multimers. The pSMNCH expression vector encodes a fusion protein containing the complete mucin amino-terminal region followed by the entire disulfide-rich domain with six histidine residues at its carboxyl terminus. In these studies, transfected cells were incubated for 15 min with [35S]cysteine-containing medium and chased with unlabeled medium, and the proteins from cell lysates and the medium were immunoprecipitated with antiserum 5287 and analyzed by SDS-gel electrophoresis and autoradiography. As shown in Fig. 7A, the predominant intracellular species migrated without prior reduction as a broad band centered at \(M_r \sim 430,000\) (lane 6), representing disulfide-bonded dimers of the protein, which on reduction gave a single protein of \(M_r \sim 220,000\). Monomeric forms were not observed, showing that dimer formation of the recombinant mucin occurs very rapidly. N-Glycosylation of the dimeric species very likely started once the protein was translocated into the endoplasmic reticulum since a second, less abundant intracellular protein, with \(M_r \sim 250,000\) on reducing gels, appeared after 15 min (lane 2) or longer chase times. Moreover, this protein was not observed when the synthesis of the N-linked oligosaccharides was inhibited with tunicamycin (data not shown). The intracellular dimers almost disappeared from the cells after 2 h of chase (lane 10). As we have reported earlier (14), dimer formation by interchain disulfide bonds between the carboxyl-terminal domains of porcine mucin to create dimers occurs very rapidly in the endoplasmic reticulum and is independent of the N-glycosylation of the monomers. Therefore, the predominant intracellular forms of the recombinant mucin observed on nonreducing SDS gels (Fig. 7A) represent dimeric species linked by interchain disulfide bonds between their carboxyl-terminal domains. Other, much less intense bands, corresponding to species of higher molecular weight, were also observed on the same nonreducing gels, suggesting the formation of disulfide-linked oligomers and multimers of the mucin protein.

As shown in Fig. 7B, on reducing SDS gels, the N-glycosylated species of \(M_r \sim 250,000\) were the major proteins purified from the medium of pSMNCH-transfected cells. Some unglycosylated species were also observed, but they disappeared from the medium after 2 h of chase (lane 5), suggesting that they were degraded. Without prior reduction, N-glycosylated dimeric species were the predominant proteins in the medium, although significant amounts of high molecular weight species that migrated in the interface between the stacking and resolving gels appeared in the medium with the same kinetics as the former. These high molecular weight forms very likely represented disulfide-linked multimers of the dimeric species that were assembled by the formation of interchain disulfide bonds among their amino-terminal regions. Consistent with this interpretation, when monensin was present in the medium (lane 10), the cells secreted only dimeric species, with no indication of multimeric forms (Fig. 7B), showing that formation of the latter occurs with a low pH. Formation of interchain disulfide bonds between the carboxyl-terminal domains occurs in the endoplasmic reticulum (14), and therefore, monensin is without effect on the synthesis of dimeric species. Formation of disulfide-linked multimers requires the cross-linking of the amino-terminal regions in the acidic compartments of the Golgi complex, a reaction that can be disrupted by monensin (Fig. 5).

Taken together, these observations suggest that dimer formation via the carboxyl-terminal domains is an early processing step that occurs rapidly after the synthesis of the mucin in the endoplasmic reticulum, consistent with studies reported earlier (14). Multimer formation via the amino-terminal domains, on the contrary, occurs in COS-7 cells once the dimers are N-glycosylated and shortly before the recombinant mucin is secreted into the medium since intracellular accumulation of multimeric species was not observed (Fig. 7A). These results also show that whereas all the precursors form dimeric species in the endoplasmic reticulum, only a fraction of the dimers are assembled into disulfide-linked multimers in the distal, acidic compartments of the Golgi complex. Thus, dimers and multimers, but not monomers, are secreted into the medium.

**DISCUSSION**

The studies reported here show that the amino-terminal region of porcine submaxillary mucin is secreted from transiently transfected cells as a single protein that forms disulfide-linked trimers. Two different antisera against polypeptides from the amino-terminal region of the mucin, in addition to absorption of the carboxyl-terminal poly-His tag with TALON-IMAC beads, failed to show proteins of lower molecular weight.
Porcine Submaxillary Mucin Multimers

FIG. 8. Schematic representation of the sequence of events in porcine submaxillary mucin biosynthesis. Step 1, porcine apomucin is synthesized in endoplasmic reticulum-bound polyribosomes and then translocated and folded in the endoplasmic reticulum. Step 2, immediately after its synthesis, the apomucin molecules dimerize in the endoplasmic reticulum through interchain disulfide bonds between the carboxyl-terminal domains including 11 half-cystines. Step 3, disulfide-bonded dimers are N-glycosylated and transported to the Golgi complex. Step 4, N-glycosylated dimeric mucins are initially O-glycosylated in the cis Golgi compartments. The biosynthesis of O- and N-linked oligosaccharides continues in the medial and trans Golgi compartments. Step 5, upon reaching the trans Golgi compartments, multimeric species of the mucin are assembled by interchain disulfide bonds involving one or more of the amino-terminal D-domains of the mucin. The homologous amino-terminal region of human pro-von Willebrand factor is proteolytically processed during its biosynthesis (reviewed in Ref. 30), which results in the secretion of two proteins, the propolypeptide, which includes the D1 and D2 domains, and mature von Willebrand factor. Formation of mature von Willebrand factor involves the cleavage of the peptide bond between Arg-763 and Ser-764 by furin (30, 31), a ubiquitously expressed subtilisin-like serine endoprotease. Proteolytic processing of human pro-von Willebrand factor has been shown to occur not only in endothelial cells, but in different cell types including COS cells (30). These observations, together with the absence of proteolytic forms of the amino-terminal region of the mucin expressed and secreted by COS-7 cells or MOP-8 cells (Figs. 1 and 2) and the fact that none of the predominant consensus recognition sequences for furin (Arg-X-Arg/Lys-Arg; e.g. Ref. 31) are found in the submaxillary mucin (1), strongly argue against a role for furin during the biosynthesis of the porcine mucin.

The amino-terminal region of the mucin expressed in COS-7 cells or MOP-8 cells forms disulfide-linked oligomers (Fig. 2). The molecular weight of these oligomers could not be determined exactly, but by SDS-gel electrophoresis, they migrated slightly slower than dimeric fibronectin (Fig. 2B) with an estimated $M_r$ (520,000) indicating the formation of disulfide-linked trimers. Consistent with this conclusion, the trimeric species also migrated slightly slower than the dimeric species of a fusion protein between the same amino-terminal region and the disulfide-rich carboxyl-terminal domain of mucin, which migrated at a similar position as dimeric fibronectin (Fig. 2B).

The mucin amino-terminal region does not form interchain disulfide bonds with the disulfide-rich carboxyl-terminal domain (Fig. 6), indicating that dimerization and trimerization are independent processes during the biosynthesis of mucin. Indeed, dimer formation via the carboxyl-terminal domains of the mucin occurs in the endoplasmic reticulum (14) and precedes multimerization through the amino-terminal domains (Figs. 3 and 7). Disulfide-linked multimers of submaxillary mucin are assembled in the Golgi complex (Fig. 4) and very likely in a compartment with a pH between 5.9 and 6.6, which is characteristic of the distal/trans compartments of the Golgi complex (Figs. 5 and 7B). These roles of the amino- and carboxyl-terminal domains of the mucin in multimer formation are consistent with their amino acid sequence similarities to the corresponding amino- and carboxyl-terminal regions of human von Willebrand factor (1). This human blood-clotting factor also forms multimers by interchain disulfide bonds among the amino-terminal regions of dimeric subunits (3, 30). Moreover, multimer formation of pro-von Willebrand factor requires a low pH and seems to occur in distal compartments of the Golgi complex. Human von Willebrand factor catalyzes its own multimerization, which depends on the presence of the D1 and D2 domains (32). Three CGLCG sequence motifs, two in the D1 and D2 domains and one in the D3 domain, resemble the active site of protein-disulfide isomerase and are thought to play an important role in the catalysis of interchain disulfide bond formation in human pro-von Willebrand factor (33). Porcine submaxillary mucin contains two CGLCG sequence motifs, one in the D1 domain and the other in the D3 domain (1), suggesting that they may also function in mucin multimerization. All the interchain disulfide bonds in mature von Willebrand factor dimeric species. Step 6, presumably, some of the glycosylated dimeric and multimeric species of the mucin are taken in the trans Golgi network by small transport vesicles, whereas the majority are concentrated in large secretory granules. Steps 7 and 8, transport vesicles and mucin granules are secreted by constitutive and regulated exocytotic pathways, respectively, into the extracellular space.
multimers are between D3 domains on different subunits, in which half-cystine 379 (34) and one or more of the half-cystines at residues 459, 462, and 464 (35) are forming interchain disulfide bonds. These half-cystines are conserved in the corresponding D3 domains of porcine submaxillary mucin (1), and they may also form interchain disulfide bonds.

Earlier studies (14, 36–38) and the studies presented here reveal the major steps that occur during submaxillary mucin biosynthesis, as summarized diagramatically in Fig. 8. Porcine apomucin is synthesized in the endoplasmic reticulum of mucous cells (36) and rapidly forms disulfide-linked dimers between its carboxy-terminal domains (14) including 11 half-cystines (37). Among the latter, one or more of half-cystines 13223, 13244, and 13246 are required for forming interchain disulfide bonds (37). N-Glycosylation of the amino- and carboxy-terminal domains of the mucin seems to occur post-translationally (Figs. 3A and 7A). N-Glycosylation is not required for dimer formation (14) or, later, for multimerization (Fig. 2), although the extracellular half-life of the unglycosylated mucin domains seems to be decreased (Figs. 1B and 7A). O-Glycosylation commences in the cis Golgi compartments (36, 38) and continues in the medial and trans Golgi compartments. Upon reaching the distal/trans compartments of this organelle, multimerization through the amino-terminal regions containing the D-domains is very likely triggered by the intraluminal acidic pH (Fig. 5A). Indeed, the action of the vacuolar H^+-ATPase is critical for trimer formation of the mucin D-domains expressed in COS-7 cells (Fig. 5B). Multimerization does not affect all the dimeric species (Fig. 7B), and eventually, dimers and trimers are constitutively secreted into the medium, the only route in COS-7 cells, or presumably accumulated in secretory granules of mucus cells (36). It is possible that secreted dimers of the mucin are assembled into disulfide-linked multimers following changes in the extracellular pH.

As a direct consequence of dimerization via the carboxy-terminal domains and trimer formation through the amino-terminal regions, it can be predicted that the porcine mucin is able to form branched multimers (Fig. 8). Multimeric species of human von Willebrand factor are built by the independent dimerization of the amino- and carboxy-terminal regions, resulting in the formation of unbranched linear structures (39). Moreover, electron microscopy studies on purified cervical, respiratory, and gastric mucins (e.g. Ref. 40) show that these mucins form flexible, long filaments of variable length. Unfortunately, the primary structure of the latter mucins is incomplete. These observations suggest that the mechanisms for the formation of disulfide-linked multimers in von Willebrand factor, porcine submaxillary mucin, and likely other mucins are similar, but not necessarily identical.

The present studies and those on the multimerization of human prepro-von Willebrand factor clearly show that the D-domains found in the amino-terminal regions of these proteins serve to form disulfide-linked multimers. It can be anticipated that similar D-domains present in the amino-terminal regions of frog integumentary mucin FIMB.1 (16) and human mucins MUC2 (17) and MUC5AC (41, 42) also serve to form interchain disulfide bonds among the respective proteins. Indeed, other studies have suggested that human MUC2 mucin forms disulfide-linked multimers (43).