Porcine Submaxillary Mucin Forms Disulfide-bonded Dimers between Its Carboxyl-terminal Domains*

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COS-7 cells transfected with three different expression vectors encoding the 240-amino acid residue, disulfide-rich domain at the carboxyl terminus of porcine submaxillary mucin have been used to determine the possible function of the domain in forming higher oligomers of the mucin polypeptide chain. The domain is expressed as a disulfide-bonded dimer, as shown by SDS-gel electrophoretic analysis of the immunoprecipitated domain in the presence and absence of reducing agent and the cross-linking agent bi(sulfosuccinimidyl) suberate. Molecular weight determination by gel filtration on agarose columns in 6 M guanidine HCl confirmed dimer formation. However, the domain expressed is heterogeneous as the result of different extents of glycosylation. Pulse-chase studies with the 35S-labeled domain show that dimer formation and secretion from cells occur very rapidly. Moreover, dimer formation is not dependent on the N-linked oligosaccharides on the domain. Evidence is presented that dimer formation most likely occurs in the endoplasmic reticulum before complex-type oligosaccharide synthesis is completed. Neither brefeldin A nor tunicamycin interferes with the rate of dimer formation. These studies suggest that the disulfide-rich domain acts to form dimers of the polypeptide chain of mucin. This role of the domain is consistent with its amino acid sequence similarity to the disulfide-rich domain of human prepro-von Willebrand factor, which also serves to form dimers of this blood coagulation factor.

Porcine submaxillary mucin has a disulfide-rich domain of ~240 residues at its carboxyl terminus (1). This domain contains 30 half-cystine residues and no free thiol groups,1 and 240 residues at its carboxyl terminus (1). This domain is expressed as a disulfide-bonded dimer, as shown by SDS-gel electrophoretic analysis of the immunoprecipitated domain in the presence and absence of reducing agent and the cross-linking agent bis(sulfosuccinimidyl) suberate. Molecular weight determination by gel filtration on agarose columns in 6 M guanidine HCl confirmed dimer formation. However, the domain expressed is heterogeneous as the result of different extents of glycosylation. Pulse-chase studies with the 35S-labeled domain show that dimer formation and secretion from cells occur very rapidly. Moreover, dimer formation is not dependent on the N-linked oligosaccharides on the domain. Evidence is presented that dimer formation most likely occurs in the endoplasmic reticulum before complex-type oligosaccharide synthesis is completed. Neither brefeldin A nor tunicamycin interferes with the rate of dimer formation. These studies suggest that the disulfide-rich domain acts to form dimers of the polypeptide chain of mucin. This role of the domain is consistent with its amino acid sequence similarity to the disulfide-rich domain of human prepro-von Willebrand factor, which also serves to form dimers of this blood coagulation factor.

Porcine submaxillary mucin has a disulfide-rich domain of ~240 residues at its carboxyl terminus (1). This domain contains 30 half-cystine residues and no free thiol groups,1 and unlike the highly O-glycosylated portions of the polypeptide backbone, it appears to be a globular structure. Such disulfide-rich domains are characteristic of many mucins and have been found in a bovine submaxillary gland mucin-like protein (2); human intestinal mucin, designated MUC2 (3); human tracheobroncheal mucin (4); rat intestinal mucin (5); and frog mucin (6). Of these mucins, the complete amino acid sequence has been established for only human intestinal mucin. This intestinal mucin contains a single disulfide-rich domain at its carboxy terminus, which is similar in sequence to each of the three contiguous disulfide-rich domains near its amino terminus (7). There is a high degree of identity in the amino acid sequences among the disulfide-rich domains from different mucins, especially in the location of the half-cystines. Moreover, there is a striking sequence identity between the disulfide-rich domains of human intestinal mucin and the four disulfide-rich domains of human prepro-von Willebrand factor (7). In addition, the four domains of prepro-von Willebrand factor and human intestinal mucin are located in the same regions of the molecule, one near the carboxyl terminus and three near the amino terminus. Although the sequence identity between the porcine submaxillary gland mucin domain and human prepro-von Willebrand factor is only ~20% (1) and thus of questionable significance statistically, the location of the half-cystines in these domains is very much the same.

The disulfide-rich domains of von Willebrand factor have been shown to form interchain disulfide bonds among von Willebrand factor monomers. The disulfide bonds between the carboxyl-terminal domains of von Willebrand factor permit dimer formation (8, 9), whereas those in the amino terminus permit multimer formation (8, 10, 11). In view of the structural similarities between von Willebrand factor, human intestinal mucin, and porcine submaxillary mucin, it was of interest to determine whether the mucin domains serve to form dimers or oligomers. Dimer or oligomer formation in mucins would be difficult to determine, however, because mucins have very high molecular weights (>10⁶) and high carbohydrate contents (75–90% carbohydrate by weight) that prevent structural analysis by methods such as gel electrophoresis or ultracentrifugation by sedimentation equilibrium.

We report here studies showing that porcine submaxillary mucin can very likely form dimers between its carboxyl-terminal domains. This was determined by expression of the disulfide-rich domain in mammalian cells transfected with cDNA encoding the domain, but devoid of the other structures of mucin that interfere with gel electrophoretic analysis. By these means, it is shown that the disulfide-rich monomer is synthesized and converted rapidly to a disulfide-linked dimer in the endoplasmic reticulum before secretion of the dimer into the medium.

EXPERIMENTAL PROCEDURES

Antiserum to the Disulfide-Rich Domain of Mucin—The antiserum (3814) specific for the mucin disulfide-rich domain was raised against a carboxyl-terminal polypeptide (residues 907-1150; Ref. 1) expressed in Escherichia coli by a glutathione S-transferase fusion expression system. A cDNA PstI/EcoRI fragment encoding the entire domain was obtained from the plasmid pPSM1A (1) and subcloned into a modified pGEX-KT vector. Expression of the latter construct in E. coli DH5α yielded a fusion protein consisting of glutathione S-transferase and the mucin domain, which was purified from crude extracts by affinity chromatography on glutathione-agarose (12). The fusion protein was cleaved with thrombin and absorbed to glutathione-agarose following the procedure of Guan and Dixon (13). The disulfide-rich domain was not adsorbed to the glutathione-agarose from the adsorbent and was used as antigen to produce rabbit antiserum 3814 following standard

1. N. Swamy, A. E. Eckhardt, and R. L. Hill, unpublished observations.
protocols from our laboratory (1). A rabbit antiserum against a peptide from the carboxyl-terminal disulfide-rich domain of pig submaxillary mucin (residues 1126–1137) was described earlier (1).

Construction of Expression Vectors—An EcoRI cDNA fragment encoding the entire mucin disulfide-rich domain was subcloned into the SV40-based eukaryotic expression vector pPROTA (14). The protein A coding sequences were removed by digestion of pPA-MC1 with PstI and XbaI, and a new multicloning site was created by inserting the following annealed oligonucleotide PstI/XbaI linkers: 5′-ATGGCAGTGAAGAAGGTCGACGAAATTCTTT-3′ and 5′-CTGAA-AGAATTCGACCTTCTTCACTGCCATGC-3′. The resulting plasmid was digested with SalI and self-ligated to produce the expression vector pMC. This plasmid encodes a fusion protein containing the trans signal peptide of 25 amino acids, three residues (GRR) derived from the new multicloning site, and the mucin disulfide-rich domain. The expression vector pMC was constructed by simultaneously digesting pMC1 and specificity-determining domain linker oligonucleotides 5′-ATGGCAGTGAAGAAGGTCGACGAAATTCTTT-3′ and 5′-CTGAAAGAATTCGACCTTCTTCACTGCCATGC-3′. The resulting plasmid encodes a protein that only differs from the protein encoded by pMC1 in that it contains six consecutive His residues just before the stop codon. All constructions were characterized by extensive restriction nuclease analysis and partial DNA sequencing by the dyeex method using the Sequenase Version 2.0 system (U.S. Biochemical Corp.).

Cell Culture and DNA Transfection—COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 20 mM glutamine, and 0.1% (v/v) streptomycin/penicillin antibiotics. NIH-3T3 cells were grown in Dulbecco's modified Eagle's medium containing 10% calf serum, glutamine, and antibiotics. Transfer of DNA into cells was performed by liposome-mediated transfection using Lipofectin (Life Technologies, Inc.) as directed by the manufacturer. COS-7 cells were grown for 48 h post-transfection before use. For stable transfection of NIH-3T3 cells, a 1:20 mixture of pBAPr1neo (17) and pMC was used. Positive colonies, selected in 0.5 mg/ml Geneticin (G418), were pooled and expanded in G418-containing culture medium. All culture reagents were from Life Technologies, Inc.

Metabolic Labeling and Purification of Recombinant Proteins—Cells at 90% confluency in 100-mm diameter culture dishes were incubated for 10 h in 10% fetal bovine serum in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% dialyzed fetal bovine serum and then labeled with 100 µCi/ml Tran35S-label (ICN Pharmaceuticals, Inc.). In some experiments, cells were subsequently washed in cold phosphate-buffered saline, pH 7.5, and chased in culture medium containing 0.24 µg/ml cycloheximide and 0.15 µg/ml methionine. At selected intervals, cells were washed with cold phosphate-buffered saline and lysed at 4°C in 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (1 mM lissi buffer). After centrifugation, the supernatant solutions were treated with preimmune serum (20 µl/ml) and 100 µl of 10% protein A-Sepharose CL-4B (Sigma) and then immunoprecipitated for 18 h at 4°C with antisera (38). Immunoprecipitates were washed with cold phosphate-buffered saline and then pelleted by brief centrifugation. Proteins secreted into the medium were immunoprecipitated after addition of concentrated lysis buffer to give a final concentration as above for cell lysates. Alternatively, proteins synthesized by cells transfected with plasmid pPROTA or pPA-MC2 were absorbed by agarose beads. Beads were then washed with cold phosphate-buffered saline and then pelleted by brief centrifugation. The conditioned medium using TALON IMAC resin (CLONTECH) following the manufacturer's directions. Briefly, the conditioned medium was made 50 mM in sodium phosphate, 10 mM Tris-HCl, pH 8.0, and incubated with TALON beads (1 ml of conditioned medium/20 µl of resin) for 30 min at 22°C. The beads were sequentially washed in the same buffer containing 100 mM NaCl and then 50 mM sodium phosphate, pH 7.0.

When used to test their effect on the biosynthesis of the recombinant proteins, tunicamycin and brefeldin A (both from Sigma) were added to the culture medium before and during metabolic labeling at final concentrations of 10 and 5 µg/ml, respectively.

Cross-linking—Poly-His-containing proteins, adsorbed to TALON resin as described above, were eluted with 25 mM sodium phosphate, pH 7.5, containing 100 mM EDTA, 100 mM sodium chloride, and 5 mM bis(sulfosuccinimidyl) suberate (Pierce) (18). After 30 min at 22°C, the column was washed extensively with an equal volume of 10 mM sodium phosphate, pH 8.3, and 50 mM sodium chloride, and the elution fraction eluted with 50 mM sodium phosphate, pH 8.3, containing 1.75% (v/v) Nonidet P-40, 1 mM phenylmethanesulfonyl fluoride or with 0.05 unit/ml endoglycosidase H (Genzyme Corp.) in 75 mM sodium acetate, pH 6.0, 1 mM phenylmethylsulfonyl fluoride (19). Reactions were stopped by mixing with an equal volume of SDS-gel electrophoresis sample buffer.

SDS-Polyacrylamide Gel Electrophoresis and Autoradiography—The proteins were eluted from their specific absorbents by boiling for 5 min in 50 µl of 50 mM Tris-HCl, pH 6.8, containing 0.5% SDS and 100 mM β-mercaptoethanol. The proteins were then reacted for 16 h at 37°C with 1 unit/ml N-glycanase (Genzyme Corp.) in 0.2 M sodium phosphate, pH 8.3, containing 1.75% (v/v) Nonidet P-40, 1 mM phenylmethanesulfonyl fluoride or with 0.05 unit/ml endoglycosidase H (Genzyme Corp.) in 75 mM sodium acetate, pH 6.0, 1 mM phenylmethylsulfonyl fluoride (19). Reactions were stopped by mixing with an equal volume of 2× SDS-gel electrophoresis loading buffer and resolved on a discontinuous polyacrylamide gel (20). A mixture of 14C-labeled proteins (Sigma) were used as molecular weight standards. This mixture includes α-lactalbumin (M, = 14,200), carbonic anhydrase (29,000), chicken egg albumin (45,000), bovine serum albumin (66,000), β-galactosidase (116,000), and myosin (205,000). Radiolabeled bands were detected by autoradiography after incubation of the gels with Amplify (Amersham Corp.) according to the manufacturer's directions. The dried gels were exposed at −70°C to Eastman Kodak BioMax or X-Omat AR films.

Analytical Gel Filtration on Sepharose CL-4B—Recombinant proteins were purified from the medium of radiolabeled COS-7 cells, transfected with pPA-MC2 or pMC, by absorption to agarose beads. The proteins were eluted with 5 mM glycine, pH 10.5. or with 6 M guanidine HCl in 100 mM sodium phosphate, pH 8.6, 100 mM β-mercaptoethanol. Following overnight incubation at 22°C, the reduced samples were alkylated for 1 h with iodoacetamide and applied to a Sepharose CL-4B column (1.7 × 83 cm) equilibrated with 6 M guanidine HCl, pH 8.0, and eluted at 20 ml/h. Fractions were collected and analyzed for radioactivity. The column was calibrated with the following protein markers: β-galactosidase (M, = 116,000; Sigma), phosphorylase b (97,400; Sigma), transferrin (76,000; ICN Pharmaceuticals, Inc.), ovalbumin (45,000; Calbiochem), carbonic anhydrase (29,300; gift from Dr. C. Fierke), and cytochrome c (12,400; Sigma).

Other Methods—Native porcine submaxillary mucin (10 mg/ml), prepared in the inhibitions of protein glycosylation (14), was digested with 0.014 µl HCl, pH 2.0, and digested with 10% (v/v) chymotrypsin (Sigma) at 37°C. After 18 h, the digest was mixed with an equal volume of 100 mM Tris-HCl, pH 8.0, 1% SDS, 200 mM 2-mercaptoethanol and boiled for 5 min. One of the samples was digested with 5 units/ml N-glycanase as described above. The digest was mixed with 2 volumes of gel electrophoresis buffer and submitted to SDS-gel electrophoresis, and the proteins were electrophoresed at constant voltage to polyvinylidene difluoride membranes (Millipore Corp.) in Towbin transfer buffer (21). Membranes were blocked for 1 h using 5% bovine serum albumin in 10 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 0.05% (v/v) Tween 20 (TBST) and incubated overnight at 4°C in the primary antisera diluted 1:100 in TBST containing 2% bovine serum albumin. Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBSST) and incubated overnight at 4°C in the primary antisera diluted 1:100 in TBSST containing 2% bovine serum albumin. Following extensive washing in TBST, proteins were visualized with enhanced chemiluminescence reagents (DuPont NEN) according to the manufacturer’s directions. Kaleidoscope prestained standards (Bio-Rad) were used as molecular weight standards.

RESULTS

Expression and Secretion of Porcine Mucin Disulfide-rich Domains in COS-7 Cells—Fig. 1A shows the SDS-gel electrophoretic patterns under reducing conditions of the proteins isolated from the medium of COS-7 cells transfected with three different expression plasmids encoding the carboxyl-terminal disulfide-rich domain of porcine submaxillary mucin. The proteins ex-
pressed by two of the plasmids, pMC and pMCH, were especially alike and showed five different species varying in molecular weight from 33,000 to 47,000. The two plasmids differed from one another by a polynucleotide in pMCH that differed from one another by a polynucleotide in pMC that encoded six histidine residues just before the stop codon. The proteins were then immunoprecipitated from the medium with antisera 3814 and analyzed by SDS-gel electrophoresis in β-mercaptoethanol and by autoradiography. B, monolayers of COS-7 cells transfected with plasmid pMC were incubated for 3 h in medium in the absence (lane 1) or presence (lane 2) of 10 μg/ml tunicamycin and labeled with Tran35S-label. Lane 1 shows the proteins without further treatment, and lane 2 shows the proteins after digestion with 1 unit/ml N-glycanase. D, purified native submaxillary mucin was digested with 10% pepsin at 37 °C for 18 h. A fraction was then reacted at pH 8.3 with 5 units/ml N-glycanase (lane 2) for 16 h at 37 °C. Proteins were separated by reducing SDS-gel electrophoresis, blotted onto polyvinylidene difluoride membranes, reacted with an antiserum against a peptide from the carboxyl-terminal mucin domain, and with a peroxidase-linked anti-rabbit antibody, and visualized by enhanced chemiluminescence. Lane 1 is the peptic digest without N-glycanase treatment.

cells secreted a single species with an Mr of 33,000, which is consistent with the conclusion that the five species differ in the extent of N-glycosylation. When the secreted proteins were isolated from the medium of transfected COS cells and treated with N-glycanase, the major band with an Mr of 33,000 was observed (Fig. 1C). To determine whether the disulfide-rich domain in native mucin contains N-linked oligosaccharides, native mucin was digested with pepsin, and electroblots of the digests were analyzed with antibodies specific for a synthetic peptide unique to the disulfide-rich domain. As shown in Fig. 1D, the antisera reacted with protein species with an Mr of ~41,000 and a broad band centered at Mr = 17,900. Components of the broad band decreased in size after treatment with N-glycanase, indicating that N-linked oligosaccharides are present in the disulfide-rich domain of native mucin. The minor bands at the top of the gels in all panels of Fig. 1 are at the interface between the stacking and running gels. These bands were also observed on gels analyzed with preimmune serum and are of unknown nature.

Formation of Dimers of the Carboxyl-terminal Disulfide-rich Domain of Mucin—Fig. 2A shows the SDS-gel electrophoretic patterns of the immunoprecipitated 35S-labeled proteins expressed and secreted into the medium of COS-7 cells trans-
Disulfide-bonded Dimers of Porcine Mucin

The purified domain was analyzed under nonreducing conditions or after reduction in β-mercaptoethanol and alklylation with iodoacetamide. The samples were applied and eluted in 6 M guanidine HCl, pH 5. The amount of eluate that emerged from the column was measured from the weight of the fractions eluted. Radioactivity (open circles) was used to detect the protein in the eluates. The protein standards (closed circles) were analyzed in the same way after reduction and alklylation with iodoacetamide and were detected in the eluates by their absorbance at 280 nm. Further details are given under “Experimental Procedures.”

The major protein on reducing gels is the monomeric species which encodes the fusion protein of the domain and protein A. The proteins expressed by COS cells transfected with pPA-MC2, under nonreducing conditions, and cross-linked dimers stable to reducing conditions, and monomers under reducing conditions. Similarly, the protein from COS cells transfected with pMCH, pPROTA, or pPA-MC2. The purified domain was found to be nonreducing in the presence of β-mercaptoethanol (lane 1), five bands were observed, as in Fig. 1A, with Mr values of 33,000–47,000. When the proteins from the medium were cross-linked with bis(sulfosuccinimidyl) suberate and analyzed on gels in the presence of β-mercaptoethanol, a broad band was observed of cross-linked proteins with a molecular weight centered at ~83,000, or about twice that of the proteins in β-mercaptoethanol. The uncrossed-linked species in the absence of β-mercaptoethanol gave a broad band of similar size (lane 3) as the cross-linked species. All gels showed a 35S-labeled band at the interface between the stacking and running gels, but this band was also found with preimmune serum (lanes 4–6). The preimmune serum, however, did not show the other bands. These results are consistent with formation of a disulfide-linked dimer of the disulfide-rich domain.

Fig. 2B shows the same type of experiment as in Fig. 2A, except that the expression plasmid pMCH was used to transfet COS cells and the expressed protein was isolated on a TALON IMAC absorbent. The proteins expressed had about the same molecular weights as those in Fig. 2A, with an Mr of 86,000–90,000, whereas that on nonreducing gels has an Mr of 180,000. Thus, formation of dimers is not influenced by the non-mucin portion of the fusion protein.

Fig. 3 shows the estimated molecular weights for the proteins expressed and secreted from transfected COS cells as judged by gel filtration on Sepharose CL-4B in the presence of guanidine HCl. These analyses confirmed the molecular weight estimates made from SDS-gel electrophoresis. Thus, the protein expressed by pPA-MC2 under nonreducing conditions had an Mr of 150,000 as compared with an Mr of 82,000 under reducing conditions. Similarly, the protein from COS cells transfected with pMCH had an Mr of 66,000 in the absence of reducing agent and an Mr of 35,000 in the presence of reducing agent. The molecular weight of pPROTA, the protein expressed by the fusion protein vector without the disulfide-rich domain insert, was about the same in the presence and absence of β-mercaptoethanol, which also indicates that dimer formation is not dependent on the non-mucin portion of the fusion protein.

Rate of Formation of the Disulfide-rich Domain Dimers—Pulse-chase studies of the formation of the dimers with 35S-labeled amino acids were used to judge the rate of dimer formation and the subcellular compartments where formation occurred. Fig. 4A shows the SDS-gel electrophoretic patterns of the proteins from COS-7 cells transfected with pMCH when radiolabeled for 10 min and then chased with unlabeled medium over time. The proteins present in the cell lysates were immunoprecipitated with preimmune serum (PI) or reduced (lanes 1–9) or reducing (lanes 10–18) SDS-gel electrophoresis. Proteins absorbed by the preimmune serum (PI) are shown in lanes 8, 9, 17, and 18. B, pMC-transfected COS-7 cells were subjected to pulse and chase as described for A. The proteins in the cell lysates were immunoprecipitated with preimmune serum (PI) and divided into two fractions, one of which was reacted with 0.05 unit/ml endoglycosidase H (Endo H) (+ lanes) for 16 h at 37°C. The proteins were analyzed by reducing SDS-gel electrophoresis and autoradiography.
from the cells. Thus, in the study shown in Fig. 4A, dimers were almost absent from cells after 2 h of chase and appeared almost exclusively in the medium at this time. A major protein band was also observed in Fig. 4A at the interface between the stacking and running gels under nonreducing conditions in both cells and media. This species was also detected with pre-immune serum, consistent with the results shown in Fig. 2. A much fainter band was observed at this position under reducing conditions. Thus, this high molecular weight species is not thought to be the result of nonspecific aggregation of mucin.

As shown in Fig. 4B, the proteins expressed in the cells transfected with pMC were sensitive to endoglycosidase H digestion between 0 and 10 min under the conditions of the chase in unlabeled medium. This result suggests that dimer formation occurs before N-linked oligosaccharides are converted to the complex type of oligosaccharides that are endoglycosidase H-resistant. Because the enzymes that catalyze the conversion of endoglycosidase H-sensitive to endoglycosidase H-resistant species exist in the medial/trans-Golgi complex (22), dimer formation must occur in the endoplasmic reticulum or the cis-Golgi apparatus. However, the endoplasmic reticulum is the main organelle acting in mucin dimer formation because cells transfected with pMC express dimers of the domain in the presence of brefeldin A (Fig. 5), a drug that disorganizes the Golgi apparatus (23). Moreover, in brefeldin A, the dimers are not secreted into the medium, nor are their N-linked oligosaccharides converted to the complex type.

Dimer formation is not specific to expression in COS-7 cells since stable transfectants of 3T3 cells with plasmid pMC also express dimers, as shown in Fig. 6. In the pulse-chase study shown, dimers (M_r = 66,000) were observed in cell lysates between 0 and 10 min of chase, but had disappeared from the cells after 120 min when they were observed in the medium. Fig. 6 also shows the large band at the interface between the stacking and running gels and another band at M_r = 55,000. These bands also appeared with pre-immune serum as in Figs. 2, 4, and 5, but are of unknown nature.

The observation that tunicamycin (Fig. 1B) does not inhibit the secretion of the disulfide-rich domain from COS-7 cells indicates that the N-linked oligosaccharides are not required for either intracellular trafficking or secretion from the cell. The pulse-chase studies in Fig. 7 show that the N-linked oligosaccharides are also not required for dimer formation. In these studies, transfected COS-7 cells were incubated with tunicamycin and then pulsed for 10 min with 35S-labeled medium and chased with unlabeled medium. The nonglycosylated proteins expressed intracellularly and secreted into the medium were dimers (M_r = 54,000) on nonreducing gels and monomers (M_r = 33,000) on reducing gels. As in Figs. 4–6, the major band on nonreducing gels at the interface between the stacking and running gels was also observed with pre-immune serum.

**DISCUSSION**

The studies reported here show that the disulfide-rich domain at the carboxyl terminus of porcine submaxillary mucin is synthesized rapidly as a dimer in transiently expressed COS-7 cells (Fig. 1) and stably expressed 3T3 cells (Fig. 6) transfected with expression vectors encoding the domain. The domain is expressed as disulfide-bonded dimers as judged by SDS-gel electrophoresis in the presence and absence of beta-mercaptoethanol and the cross-linking agent bis(sulfosuccinimidyl) suberate (Fig. 2). The domain contains four Asn-Thr/Ser sequences that are N-glycosylated to different extents during dimer formation (Fig. 1). Although native mucin contains N-linked oligosaccharides in the disulfide-rich domain (Fig. 1D), the oligosaccharides are apparently not involved in dimer formation. The differential glycosylation of the domain observed in COS-7 cells is not necessarily a feature of domain formation and likely reflects the high rate of expression of the domain and overloading the glycosylation capacity of transfected COS cells. Indeed, the dimers formed in stably transfected 3T3 cells (Fig. 6) are more extensively glycosylated than those in COS cells. Although tunicamycin inhibits the glycosylation, maturation, and secretion of rat gastric mucin (24), there is no evidence for such effects in dimer formation of the expressed disulfide-rich domain.

Pulse-chase studies (Figs. 4–6) indicate that the dimers of the disulfide-rich domain are formed rapidly once protein synthesis commences. Dimer formation most likely occurs in the endoplasmic reticulum since the first dimers detected (Fig. 4B) are susceptible to endoglycosidase H digestion. This result is consistent with the observation that rat gastric mucin (24) oligomers form in the endoplasmic reticulum, where most secretory proteins are folded and assembled into oligomers (re-
Disulfide-bonded Dimers of Porcine Mucin

![Graph showing SDS-gel electrophoresis results](image)

**Fig. 7.** Synthesis and secretion of the disulfide-rich domain of mucin in the presence of tunicamycin. COS-7 cells transfected with plasmid PMC were incubated for 3 h with 10 μM tunicamycin in the culture medium and then subjected to pulse-chase analysis as described for Fig. 4A in the presence of tunicamycin. Proteins from cell lysates (C) or medium (M) were immunoprecipitated with antisemur 3814 and analyzed by SDS-gel electrophoresis in the absence (lanes 1-7) or presence (lanes 8-14) of μ-mercaptoethanol.

viewed in Ref. 25). That dimer formation is an early event in the endoplasmic reticulum is consistent with other secreted mucins such as human gastric mucin (26) and human gall bladder mucin (27). However, studies on these mucins are difficult to interpret because the high molecular weights of dimers and their precursors are difficult to measure accurately by gel electrophoresis, which is not a problem in the present studies that examine dimer formation of the disulfide-rich domain in the absence of other parts of the mucin polypeptide chain.

The molecular weights of the domains determined by gel electrophoresis are inexact because the mobilities of proteins are influenced markedly by the presence or absence of disulfide bonds, covalent cross-links, and N-linked oligosaccharides. However, the estimated values reported here are sufficiently accurate to leave little doubt that the domains are synthesized as disulfide-bonded dimers. Nevertheless, gel filtration in 6 M guanidine HCl was used as an independent method to check the values obtained by gel electrophoresis.

The SDS gels used to detect monomers and dimers also showed immunoreactive proteins of high molecular weight accumulating at the interface between the stacking and running gels. The amounts of protein at the interface varied somewhat from one gel to another under nonreducing conditions, and under reducing conditions, the interfacial protein was much reduced or absent. Moreover, the interfacial proteins were also observed when preimmune serum was used to precipitate the expressed proteins from cells or medium. These observations suggest that the interfacial proteins are disulfide-bonded proteins that nonspecifically react with components of rabbit serum, but not with the anti-mucin antibodies. The exact nature of the interfacial protein remains to be determined, but it may be aggregates of the domain that form nonspecifically in cells. Of interest is the fact that no evidence for very high molecular weight protein comparable to the interfacial protein was observed on gel filtration of purified proteins in 6 M guanidine HCl.

The carboxyl-terminal disulfide-rich domain of porcine mucin is marginally identical in its complete amino acid sequence to the carboxyl-terminal disulfide-rich domain of human prepro-von Willebrand factor (1). Nevertheless, the half-cystine residues have a high degree of identity. Moreover, the analogous domains from other types of human mucin that show more significant sequence identity to prepro-von Willebrand factor (7) also show statistically significant sequence identity to the porcine disulfide-rich domain. Thus, since dimer formation of von Willebrand factor is the result of disulfide bonding among its carboxyl-terminal disulfide-rich domains, a similar function for the corresponding domain in mucins is not particularly surprising. Nevertheless, proof of dimer formation in mucins would be very difficult to establish by direct analysis of native mucins because their high carbohydrate contents, and high molecular weights, prevent direct analysis by gel electrophoretic or physicochemical methods for examining proteins in solution.

Human prepro-von Willebrand factor and human intestinal mucin (MUC2) (7) have three additional disulfide-rich domains in their amino-terminal regions. These domains act in human prepro-von Willebrand factor to form disulfide-bonded oligomers. Similar domains are present in porcine submaxillary mucin, but further studies will be necessary to determine whether they serve to form oligomers in mucins.

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2 A. E. Eckhardt and R. L. Hill, unpublished observations.
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