The Carboxyl-terminal 90 Residues of Porcine Submaxillary Mucin Are Sufficient for Forming Disulfide-bonded Dimers*  

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

COS-7 cells transfected with expression vectors encoding 90 and 154 amino acid residues, respectively, from the carboxyl terminus of the disulfide-rich domain (240 residues) of porcine submaxillary mucin were shown to form disulfide-bonded dimers. Cells with expression vectors that encoded the disulfide-rich domain lacking the last 90 and 150 carboxyl-terminal residues, respectively, from the carboxyl terminus of the disulfide-rich domain were unable to secrete truncated domains. These results indicate that the information required to form disulfide-bonded dimers resides in only 90 residues, including 11 half-cystines. Site-specific mutagenesis was employed to change, one at a time, each codon for the 11 half-cystines to serine. Eight of the 11 mutants formed disulfide-bonded dimers indistinguishable from those produced by unmutated vector, although 6 of the 8 mutants also produced aggregates thought to be misfolded protein with scrambled disulfide bonds. Two additional mutant vectors encoding serine instead of half-cystine at residues 13244 and 13246 in submaxillary mucin expressed both monomers and dimers of the disulfide-rich domain but no aggregates. The final mutant vector, C13223S, expressed protein aggregates that were poorly secreted from transfected cells. A mutant vector with two codon changes, C13244A/C13246A, expressed both monomers and dimers, just like the single mutants at these half-cystines. These results suggest that three half-cystine residues (Cys13223, Cys13244, and Cys13246) may be involved in forming interchain disulfide bonds in mucin dimers. Two of these half-cystines, Cys13244 and Cys13246, are in the highly conserved sequence Cys13244LCys13246C in the disulfide-rich domain of several other human mucins and in prepro-von Willebrand factor and norrin. 

Porcine submaxillary mucin contains a polypeptide chain of 13,288 residues, which can be divided into domains typical of those found in several other mucins secreted by various human tissues. Most of the polypeptide chain is comprised of 81 residues that are rich in serine, threonine, glycine, and alanine, and together account for 75% of the residues in this domain. There are at least three different genes encoding porcine submaxillary mucin that differ from one another in the number (90, 125, and 135) of repeats they encode (1). The tandem repeat domain is flanked on either end by unique sequences that are similar in composition to the repeat domains but exhibit no sequence identity to one another or to the tandem repeat domains. The hydroxyl groups of serine and threonine in the tandem repeat domain (2), and presumably the unique sequences flanking this domain, are in O-glycosidic linkage with oligosaccharides, which account for about 75% of the weight of native mucin. Three disulfide-rich domains are at the amino terminus of submaxillary mucin (1). These domains show considerable sequence identity to the D-domains found in T.E.C.H.a prepro-von Willebrand factor (3), frog integumentary mucin FIMB.1 (4), and a human intestinal mucin (MUC2) (5). The carboxyl terminus of submaxillary mucin is formed by a 240-residue disulfide-rich domain, which has significant sequence identity with similar domains at the carboxyl terminus of human prepro-von Willebrand factor (3), frog integumentary mucin FIMB.1 (4), and human mucins secreted by different tissues, including MUC2, MUC5AC (4), MUC5B (6), and MUC6 (5). Moreover, the 11 half-cystines in a 133-residue protein (norrin) associated with Norrie disease in humans (11, 12) are conserved in the carboxyl-terminal disulfide-rich domains of human prepro-von Willebrand factor, porcine submaxillary mucin, and several human mucins. It has been shown that the disulfide-rich domain of prepro-von Willebrand factor (14), porcine submaxillary mucin (15), and norrin (16) forms interchain disulfide bonds between two polypeptide chains in these molecules.

We describe here studies designed to determine whether the entire 240-residue carboxyl-terminal disulfide-rich domain is required for the formation of disulfide-linked dimers of porcine submaxillary mucin. The approach taken was to mutate plasmids encoding the domain by deletion of segments of DNA from either the 5' or the 3' end and to examine the ability of the truncated plasmids when transfected into COS-7 cells to form disulfide-bonded dimers. Further studies by site-specific mutagenesis permitted determination of those half-cystine residues that appear to form the interchain disulfide bonds in dimers.

EXPERIMENTAL PROCEDURES

Construction of Mutant Expression Vectors—Site-specific mutagenesis was achieved as described earlier (17) with reagents from CLONTECH and, as template, the expression vector pMC (15), which encodes the 244 residues of the disulfide-rich carboxyl-terminal residues of porcine submaxillary mucin (15), and norrin (16) forms interchain disulfide bonds between two polypeptide chains in these molecules.

Porcine submaxillary mucin contains a polypeptide chain of 13,288 residues, which can be divided into domains typical of those found in several other mucins secreted by various human tissues. Most of the polypeptide chain is comprised of 81 residues that are rich in serine, threonine, glycine, and alanine, and together account for 75% of the residues in this domain. There are at least three different genes encoding porcine submaxillary mucin that differ from one another in the number (90, 125, and 135) of repeats they encode (1). The tandem repeat domain is flanked on either end by unique sequences that are similar in composition to the repeat domains but exhibit no sequence identity to one another or to the tandem repeat domains. The hydroxyl groups of serine and threonine in the tandem repeat domain (2), and presumably the unique sequences flanking this domain, are in O-glycosidic linkage with oligosaccharides, which account for about 75% of the weight of native mucin. Three disulfide-rich domains are at the amino terminus of submaxillary mucin (1). These domains show considerable sequence identity to the D-domains found in T.E.C.H.a prepro-von Willebrand factor (3), frog integumentary mucin FIMB.1 (4), and a human intestinal mucin (MUC2) (5). The carboxyl terminus of submaxillary mucin is formed by a 240-residue disulfide-rich domain, which has significant sequence identity with similar domains at the carboxyl terminus of human prepro-von Willebrand factor (3), frog integumentary mucin FIMB.1 (4), and human mucins secreted by different tissues, including MUC2, MUC5AC (4), MUC5B (6), and MUC6 (5). Moreover, the 11 half-cystines in a 133-residue protein (norrin) associated with Norrie disease in humans (11, 12) are conserved in the carboxyl-terminal disulfide-rich domains of human prepro-von Willebrand factor, porcine submaxillary mucin, and several human mucins (13). It has been shown that the disulfide-rich domain of prepro-von Willebrand factor (14), porcine submaxillary mucin (15), and norrin (16) forms interchain disulfide bonds between two polypeptide chains in these molecules.

We describe here studies designed to determine whether the entire 240-residue carboxyl-terminal disulfide-rich domain is required for the formation of disulfide-linked dimers of porcine submaxillary mucin.

† To whom correspondence should be addressed: Dept. of Biochemistry, P.O. Box 3711, Duke University Medical Center, Durham, NC 27710. Tel.: 919-681-8805; Fax: 919-684-5040; E-mail: hill@biochem.duke.edu.

‡ GenBankTM accession number X04385.

§ GenBankTM accession number X04385.

** GenBankTM accession number Z48314.

† GenBankTM accession number Y09788.

GenBankTM accession number U97698.

GenBankTM accession number Y09788.

GenBankTM accession number Z48314.

GenBankTM accession number Y09788.

GenBankTM accession number Z48314.

GenBankTM accession number U97698.
mucin. Primers were synthesized by the Duke University Oligonucleotide Synthesis Facility or by Life Technologies, Inc. The selection primer was the antisense oligonucleotide, 5'-CTGCAGTCTGCTTCCTGTTCACTC-3', which initiates a silent mutation that destroys a unique SalI site in the multicloning site of pmC. To construct the vector pmCbc, a SalI/SpeI site was created by inserting a fragment containing the truncated mucin domain, residues 13131–13288 (1). The antisense mutagenic oligonucleotides used for substitutions of cysteine to another amino acid in pmC were as follows: C13200S, 5'-CACGCGGGAAGGTTTGGAACTACTCCCTTCC-3'; C13214S, 5' -CTTTGCAGGGAAGGCTTTCCTACGAGG-3'; C13246S, 5'-CTTGCCGAGGGAAGGACTATTTGCTTC-3'; C13247S, 5'-CTCTCTTGGGGCCAAACAGCATG-3'; C13261S, 5'-CACTCAGGAGTCTCAGAACTATC-3'; C13275S, 5'-CTAAAGCAGGACCGGCTTCTGTC-3'; C13279S, 5'-CAGGATCCATTAGAGGACCGC-3'; C13255S, 5'-GATGTGTTGGAGGGCTCAACAGG-3'; C13258S, 5'-GGACGCCAAGAGATGTTTTTCAACTG-3'; C13246S, 5'-CTTGCCGAGGGAAGGACTATTTGCTTC-3'; C13247S, 5'-CTCTCTTGGGGCCAAACAGCATG-3'; and C13244A, 5'-CTGTTGGCACAGCAGCATGTTTTTCAACTG-3'.

The vector pMCA was made by introducing into pmC a SpeI site downstream to the Gly13213 codon with the following antisense mutagenic primer, 5'-TTTTTCACAGTGTCTCGAGTTTC-3'. A Xhol/SpeI mutant-encoding polynucleotide was then obtained from the resulting plasmid and subcloned between the SalI and the Spel sites of pmC. The final expression vector encodes a fusion protein containing a 25-residue signal peptide (16). Residues derived from the cloning site consist of 9 residues at the carboxyl terminus of mucin (residues 13198–13288) and 6 residues of the cloning site (GRRILSR), followed by 90 residues encoding the transin signal peptide of 25 residues, followed by 3 amino acids (GRE) that precede a truncated mucin domain (residues 13045–13128) and the tetrapeptide LETL at the carboxyl terminus.

The expression vector pMCb was constructed by replacing the protein A coding sequences of plasmid pPA-MC1 (15) with a new multicloning site as described earlier (15). The resulting plasmid was then digested with XhoI and Spel and ligated to a Xhol/Spel mucin DNA fragment encoding residues 12572–12928 (1). A new XbaI site was made by site-specific mutagenesis upstream from the codon for serine residue 12572 with the following antisense mutagenic primer; 5'-GGTTTGCAGCTTCTCAGTATGGAACA-3'. The following oligonucleotide was used as selection primer: 5'-CTAGAAGGAGGCCAGGCTGACACAC-3', which destroys a unique SalI site in the multicloning site of the template plasmid. A poly-His-encoding cassette was inserted upstream to the stop codon by published procedures (15).

The resulting plasmid was digested with XbaI and ligated to give pMCb, which encodes a fusion protein containing a 25-residue signal peptide, 7 residues of the cloning site (GRRILSR), followed by 90 residues at the carboxyl terminus of mucin (residues 13198–13288) and 6 histidine residues. The Cys to Ala mutants of pMCb were constructed with the same antisense mutagenic oligonucleotides used with pmC, and the antisense selection primer 5'-GCCAATCCTTGGAGAAGATTCG-3', which destroys a unique XbaI site. To facilitate the screening process, the mutagenic oligonucleotides were designed to produce silent mutations that destroy or create restriction sites in the mucin sequence along with the desired codon changes. All mutations in the expression vectors were confirmed by DNA sequencing with the Sequenase 2.0 system from U. S. Biochemical Corp. The entire polynucleotide encoding the mutant C13223S was repeatedly sequenced to establish that no unintended mutation had been made.

Transfection of Mammalian Cells—COS-7 cells were grown and transfected with Lipofectin (Life Technologies, Inc.) as described (15) except that chloroquine, at a final concentration of 50 μm, was included in the transfection mixture. NIH-3T3 fibroblasts, three or four different lines from each, and 77 strains of COS cells were transfected with plasmid pHBApH1neo (19) and the indicated construct as reported earlier (15).

Purification and Analysis of Recombinant Proteins—Antiserum 3814 against the carboxyl-terminal disulfide-rich domain of mucin was described earlier (15). Metabolic labeling of cells with Tran35S-label or 38S-cysteine (both from ICN Pharmaceutical, Inc.) and immunoprecipitation with antiserum 3814 were performed as described earlier (15) except that immunoprecipitates were washed further with 50 mM Tris-HCl, pH 8.0, 0.25 mM NaCl containing 0.01% each of SDS, sodium deoxycholate, and Nonidet P-40. To block free thiol groups that could permit formation of incorrectly paired half-cystines during cell lysis and immunoprecipitation, intracellular precursors were reacted in vivo with iodoacetamide as reported earlier (20), and media were reacted with iodoacetamide prior to immunoprecipitation. SDS-gel electrophoresis and autoradiography were done as described earlier (15). 13C-labeled proteins (Amersham Pharmacia Biotech) were used as molecular weight standards, including myosin (220,000), phosphorylase b (97, 400–100,000), bovine serum albumin (66,000), ovalbumin (46,000–50,000), carbonic anhydrase (30,000), and lysozyme (14,300).

Detection of Free Thiol Groups—His-tagged proteins were purified from the medium of transfected cells on TALON-IMAC beads (CLONTECH) (15), eluted from the beads with the 0.5 ml of Tris, pH 8, containing 10 mM EDTA, and 6 M urea (buffering binder), and then applied to a column of activated thiopropyl-Sepharose 6B (Sigma) equilibrated with binding buffer. After 2 h at 25 °C, the column was sequentially eluted with binding buffer, the same buffer containing 1 M NaCl, and then with binding buffer-NaCl containing 10 mM dithiothreitol. Fractions (0.15 ml) were collected and counted for radioactivity. In some experiments, fractions were desalted on Sephadex G-25 (Pharmacia-LKB) in 50 mM Tris, pH 8, containing 0.15 M NaCl and 0.1% Triton X-100. The eluted lane was isolated by immunoprecipitation with antisemur 3814. Immunoprecipitates were analyzed as described earlier (15) by SDS-gel electrophoresis and autoradiography.

RESULTS

Expression and Secretion of Truncated Forms of the Carboxyl-terminal Disulfide-rich Domain of Mucin—The expression vector pMC (15) is a plasmid that encodes a fusion protein containing a 25-residue signal peptide of 25 residues followed by 3 residues (GRE) derived from a multi-cliioning site and then 244 residues that encode the disulfide-rich domain at the carboxyl terminus of mucin. As a first step to identify which of the 30 half-cystine residues are involved in formation of interchain disulfide bonds between two of the disulfide-rich domains, pMC was truncated at its amino terminus by site-specific mutagenesis. Two truncated plasmids were produced. One, designated pMCbc, is devoid of the first 86 residues of the disulfide-rich domain and encodes 158 residues (residues 13131 to 13288 in mucin) containing 20-half-cystine residues. The other, designated pMCcH is devoid of the first 154 residues of the disulfide-rich domain and encodes the last 90 residues (residues 13198 to 13288) at the carboxyl terminus of the domain, including 11 half-cystine residues, followed by 6 histidine residues, which were introduced to aid in purification of the protein expressed by the plasmid. Fig. 1A shows the electrophoretic pattern on SDS gels in 2-mercaptoethanol of the proteins immunoprecipitated from the medium of COS-7 cells that had been transfected with the truncated plasmids and metabolically labeled with Tran35S-label. The protein produced by pMCbc (lane 2) migrated as a broad band with Mr = 22,000–28,000, whereas pMCcH (lane 3) was expressed as two proteins with Mr = 17,000 and 19,000, respectively. However, if the proteins produced by these plasmids were digested with N-glycanase, only one protein species was observed on gels (Fig. 1B). pMCbc gave a protein with Mr = 22,000, and pMCcH gave a protein with Mr = 17,000. Treatment of transfected cells with tunicamycin also showed single protein species of these sizes on gel electrophoresis (data not shown). These results are similar to those obtained earlier (15) on transfection of COS-7 cells with pmC and show that the different sizes of proteins expressed by the plasmids result from different extents of N-glycosylation, with pMCbc containing two glycosylation sites and pMCcH containing one.

Fig. 1A also shows the proteins produced by COS-7 cells transfected with these plasmids and analyzed on nonreducing gels. pMCbc (lane 5) produced a protein with Mr = 48,000,
FIG. 1. Expression and secretion of truncated mutants of the disulfide-rich domain of mucin. A, COS-7 cells transfected with the expression plasmids pMCb (lanes 1, 2, 4, and 5) or pMCh (lanes 3 and 6) were metabolically labeled 48 h post-transfection with Tran35S-label for 3 h. Proteins from the medium were immunoprecipitated with preimmune serum (lanes 1 and 4) or antiserum 3914 (lanes 2, 3, 5, and 6), absorbed to and eluted from protein A-Sepharose beads, and analyzed by reducing (lanes 1–3) or nonreducing (lanes 4–6) SDS-gel electrophoresis and autoradiography. The molecular weight of the standards are in thousands. B, 35S-labeled proteins secreted by COS-7 cells transfected with vectors pMCbc (lanes 1 and 2) or pMCch (lanes 3 and 4) were purified as in Fig. 1A. The proteins were denatured by boiling in 2-mercaptoethanol in buffered SDS and then incubated with N-glycanase (lanes 1 and 3) or buffer alone (lanes 2 and 4) as described earlier (15). The digests were analyzed by SDS-gel electrophoresis and autoradiography. C, COS-7 cells were transfected with vectors pMC (lane 1), pMCa (lane 2), or pMCb (lane 3). After 48 h, the cells were incubated with Tran35S-label for 3 h, and proteins were immunopurified from the medium and analyzed by SDS-gel electrophoresis as in Fig. 1A.

about twice the size of the species observed in the presence of reducing agent. pMCh (lane 6) produced three protein bands with \( M_r = 33,000, 35,000, \) and 37,000, respectively. These results indicate that the proteins produced by the plasmids formed disulfide-linked dimers. The different protein bands observed with pMCh are the result of dimer formation among the two monomeric species observed under reducing conditions. These results also indicate that the half-cystine residues involved in forming interchain disulfide bonds in disulfide-rich domain dimers (15) are among the 11 half-cystines contained in the last 90 amino acids from the carboxyl terminus of the domain. This conclusion is consistent with the observation that transfection of COS-7 cells with 2 other plasmids, pMCab and pMCa, which lacked DNA encoding the last 90 and 150 carboxyl-terminal residues, respectively, in the disulfide-rich domain, did not secrete any proteins into the medium as judged by SDS-gel electrophoresis of immunoprecipitates (Fig. 1C).

Secretion and Dimer Formation of Half-cystine to Serine Mutants of the Disulfide-rich Domain—By site-specific mutagenesis of pMC, each of the codons for the 11 half-cystine residues contained in the last 90 residues of the disulfide-rich domain was changed one at a time to the codon for serine. The proteins expressed by each of the mutant plasmids were then determined after transfection of COS-7 cells with the plasmids followed by metabolic labeling of the cells with Tran35S-label.

immunoprecipitation of the proteins from the medium, and autoradiography of the proteins after gel electrophoresis. As shown in Fig. 2A, all mutants expressed and secreted into the medium five proteins species ranging in size from \( M_r = 33,000 \) to 47,000 on gels run in the presence of reducing agent. The five proteins differ in the extent of N-glycosylation as shown earlier (15). The amounts of protein expressed by each mutant appears to be about the same as that for pMC (lane 2) or construct encoding mutants C13283S (lane 3), C13279S (lane 4), C13247S (lane 5), C13261S (lane 6), C13244S (lane 7), C13244S (lane 8), C13200S (lane 9), or C13214S (lane 10) were purified from the media as described in Fig. 1A, and analyzed without prior reduction by SDS-gel electrophoresis and autoradiography. Proteins expressed by pMC-transfected cells and absorbed by the preimmune serum are shown in lane 1. B, 35S-labeled protein from pMC-transfected cells (lanes 1 and 2) or cells transfected with construct encoding mutants C13283S (lane 3), C13279S (lane 4), C13247S (lane 5), C13261S (lane 6), C13244S (lane 7), C13227S (lane 8), C13200S (lane 9), or C13214S (lane 10) were purified as in Fig. 1A and analyzed by SDS-gel electrophoresis without reduction as in Fig. 2B. Proteins absorbed by the preimmune serum are shown in lane 1.
C13279S (lane 4) showed more radioactivity at the interface between the stacking and running gels than the amounts seen normally with unmutated proteins (lane 2) and with preimmune serum (lane 1). This suggests that aggregates of the disulfide-rich domain are expressed by these plasmids, especially in the case of C132005S (lane 9), C13214S (lane 10), and C13247S (lane 7). The nature of these aggregates is unknown, but they likely result from incorrect formation of interchain and intrachain disulfide bonds that contain many covalently linked monomeric polypeptides.

Fig. 2C shows the autoradiographs of the proteins on nonreducing gels that were expressed by COS-7 cells transfected with two mutant plasmids not shown in Fig. 2B. The majority of the protein expressed by these mutants, C13244S (lane 4) and C13246S (lane 3), were of about the size (Mr = 73,000) as that of disulfide-bonded dimers expressed by pMC (lane 2), the unmutated plasmid. Moreover, there was no more radioactivity between the running and stacking gels than seen with the pMC (lane 2) and with preimmune serum (lane 1), indicating that they did not express aggregates. However, each mutant plasmid expressed five protein bands, when analyzed on nonreducing gels, that are characteristically seen on reducing gels (Fig. 2A). These results suggest that a small but significant amount of non-disulfide-bonded monomer is secreted into the medium of the cells transfected with these two plasmids. The unreduced monomeric species expressed by C13244S migrated slightly faster than those from C13246S.

Fig. 3A shows the autoradiographs of the proteins expressed by the remaining mutant plasmid not shown in Figs. 2, B and C. The plasmid, C13223S, was found to secrete very little protein into the medium when analyzed either on reducing (lane 1) or nonreducing (lane 3) gels. Under nonreducing conditions, the secreted (lane 3) and unsecreted (lane 4) proteins expressed by this plasmid were found at the interface of the stacking and running gels, which is characteristic of disulfide-bonded aggregates. Dimeric species, similar to those secreted by the unmutated domain, were not observed. The large majority of the protein was found in cell lysates (lanes 2 and 4) and was present in reducing gels as five species of protein (lane 2) resulting from differences in the extent of N-glycosylation. When transfected cells were radiolabeled for 4 or more hours, immunoprecipitates of the cell lysates from transfected COS-7 cells also showed a faint band with Mr = 80,000 (Fig. 3B, lane 1). This protein was neither absorbed by the pre-immune serum (lane 2) nor observed in immunoprecipitates from cells expressing the unmutated domain (lane 2). Moreover, it is not found in the medium (Fig. 3A, lane 1). The nature of this protein is not known, but it did not immunoprecipitate with antisera to BiP (GRP78) or to calnexin, chaperones (21) that could be involved in folding of the domain. Pulse-chase studies with the mutant plasmid as shown in Fig. 3C indicated that the expressed protein was still present in transfected COS-7 cells 3 h after synthesis of the protein commences, the time when they were endoglycosidase H-sensitive. This result suggests that intracellular precursors of C13223S are retained in the endoplasmic reticulum during the chase period. Proteins from unmutated plasmids that express the disulfide-rich domain were found earlier (15) to be completely secreted after 2 h. Thus, it is very likely that the mutant expressing C13223S impairs dimerization and secretion of the disulfide-rich domain of mucin.

Secretion and Dimer Formation of Half-cystine to Alanine Mutants of the Disulfide-rich Domain—The studies shown in Figs. 2 and 3 indicated that three of the half-cystine to serine mutant plasmids, C13223S, C13244S and C13246S, expressed proteins from transfected cells that were different in some respect from that expressed by the other mutant and unmutated plasmids. To determine whether an amino acid other than serine produced the same kinds of effects on the disulfide-rich domains, these three half-cystines were changed to alanine by site-directed mutagenesis, and the proteins produced were analyzed as described above. Fig. 4A shows that the proteins expressed by C13223A, C13244A, and C13246A behave exactly like the corresponding proteins that contained serine instead of alanine. The secreted forms of C13244A (lane 9) and C13246A (lane 8) showed substantial amounts of dimers on nonreducing gels but also had small but significant amounts of the five monomeric species that differ in the extent of N-glycosylation and are characteristic of the monomeric species observed on reducing gels (lanes 3 and 4). Moreover, the protein expressed by the mutant C13223A is poorly secreted from the cells (lanes 5 and 10) and is recovered from cell lysates as disulfide-bonded aggregates (lane 6), with no indication of dimeric species.

Fig. 4B shows autoradiograms of the proteins expressed by NIH-3T3 cells that were stably transfected with the mutant plasmids that have the C13244A (lane 4) and the C13246A (lane 3) mutation. The behavior of the mutant disulfide-rich domain is essentially identical to that obtained from transiently transfected COS-7 cells. Thus, the mammalian expression system appears to have no effect on the nature of the disulfide-rich domains expressed.
Site-specific mutagenesis was also used to prepare a plasmid that encoded a disulfide-rich domain with both half-cystine 13244 and 13246 changed to alanine. Fig. 4C shows SDS gel electrophoresis of the proteins secreted into the medium by COS-7 cells transfected with this plasmid and then metabolically labeled with Trans35S-label. Except for the presence of aggregates, this plasmid produced the same pattern of proteins on unreduced gels as those found when cells were transfected with plasmids with only one of either of the two codons for half-cystine changed to alanine. Dimers were the predominant species secreted, but significant amounts of the five monomeric species and some aggregates were observed (lane 2). This suggests that half-cystines other than C13244 and C13246 may be involved in formation of disulfide-bonded dimers.

Two mutants of pMCcH, which encodes the carboxyl-terminal 90 residues of the disulfide-rich domain, were also subjected to site-specific mutagenesis to attempt to gain further insight into the possible role of half-cystine 13244 and 13246 in dimer formation. Fig. 5 shows the autoradiographs of the proteins secreted into the medium by COS-7 cells transfected with mutant plasmids of pMCcH that have the C13244A or the C13246A mutation. The amounts of protein expressed by the cells transfected with the mutant plasmids were lower than those from cells transfected with normal plasmid, but the species secreted into the medium by the cells were the same, except that monomers were not found in the media of cells transfected with normal plasmid. Cells transfected with the mutant plasmids secreted dimers and monomers of the disulfide-rich 90-residue domain as observed on nonreducing gels (Fig. 5). The mutant protein produced by C13244A (lane 8) had three monomeric species on nonreducing gels. The fastest moving species may have a different pattern of disulfide bonds than the other two. A third mutant vector encoding the carboxyl-terminal 90 residues of mucin with C13244 and C13246 replaced by alanine expressed and secreted the same species as the single alanine mutants for each half-cystine (data not shown). This is further evidence that the carboxyl-terminal 90 residues of mucin encoded by this plasmid are involved in formation of disulfide bonds between proteins.

COS-7 cells transfected with plasmid pMCcH (lanes 1, 2, 5, 6, and 10), C13244A (lanes 3 and 8), or C13244A (lanes 4 and 9) incubated with Trans35S-label, 48 h post-transfection, and the proteins from cell lysates (C) or medium (Medium) were purified as in Fig. 1A. Proteins were analyzed by SDS-gel electrophoresis in the presence (lanes 1–5) or absence (lanes 6–10) of 2-mercaptoethanol. B, NIH-3T3 cells expressing the unmutated mucin domain (lane 2) or the mutant domains C13246A (lane 3) or C13244A (lane 4) were incubated in the presence of Trans35S-label. Proteins from the medium were purified as in Fig. 1A and analyzed by SDS-gel electrophoresis without prior reduction. The proteins absorbed by the pre-immune serum from the medium of cells expressing the unmutated domain are shown (lane 1). C, COS-7 cells transfected with pMC (lane 1) or a plasmid encoding the double mutant domain C13244A/13246A were metabolically labeled with Trans35S-label, and proteins were purified as described in Fig. 1A. Proteins were analyzed by nonreducing SDS-gel electrophoresis and autoradiography.
The present studies suggest that formation of disulfide-bonded dimers of the carboxyl-terminal disulfide-rich domain of porcine submaxillary mucin is dependent only on structures in the 90 carboxyl-terminal residues of mucin, including 11 half-cystines (Fig. 1). By site-specific mutagenesis, each of the codons for the 11 half-cystines was changed one at a time to serine, and the ability of the resulting mutant proteins to form disulfide-bonded dimers was examined (Fig. 2). Two of the mutants, C13227S and C13283S, were identical to the normal domain. Six others, C13200S, C13214S, C13247S, C13261S, C13277S, and C13279S, formed dimers indistinguishable from normal protein although they also formed aggregates of disulfide-bonded dimers (16). Half-cystine 13246 in submaxillary mucin (15) forms dimers, and norrin forms disulfide-linked oligomers (16). Half-cystine 13246 of porcine submaxillary mucin is in an identical position to half-cystine 2010 in norrin. Moreover, a mutant form of von Willebrand factor containing an arginine instead of half-cystine 2010 fails to form dimers (22). In addition, a mutant form of norrin containing alanine instead of half-cystine 95 has impaired oligomer formation (16). Taken together, these observations argue strongly for the involvement of half-cystine 13246 of porcine submaxillary mucin in formation of disulfide-bonded dimers. Similar support for the involvement of porcine mucin half-cystine 13244 in interchain disulfide bonds of dimers is not available, but noteworthy is the conservation of the sequence CXCC in the seven proteins shown in Fig. 7, with the three half-cystines corresponding to half-cystines 13244, 13246, and 13247, respectively, in porcine mucin. It is possible that substitutions of half-cystine 13244 result in changes in conformation that affect the interchain disulfide bonds formed by half-cystine 13246. This view is consistent with the observation that mutant proteins C13244S and C13244A migrate slightly faster on SDS-gel electrophore-
sis under nonreducing conditions than mutant proteins C13246S and C13246A (Figs. 2C, and 4, A and B).

Support for the involvement of half-cystine 13223 in formation of disulfide-bonded dimers in mucin also comes from comparison of the sequences shown in Fig. 7. Half-cystine 13223 is the first half-cystine in the sequence VEMARCVGECKK, which is one of the most highly conserved sequences in these proteins. Substitution of the half-cystine in norrin that corresponds to half-cystine 13223 by either tryptophan (23) or tyrosine (24) produces Norrie disease in humans. Moreover, when valine 60 of norrin, which corresponds to valine 13218 in submaxillary mucin, is substituted by glutamic acid, the secretion of the mutant norrin is impaired (16), and humans with this mutation have a severe form of Norrie disease (13). Valine 13218, five residues removed from half-cystine 13223, may well be in a sequence that is directly involved in dimer formation through disulfide bonds.

The recent report (16) describing the oligomerization of human norrin, in conjunction with the studies reported here, strongly suggests that this type of domain with 11 half-cystines functions to form interchain disulfide bonds. This view is supported by the recent report (10) that human MUC6 contains a carboxyl-terminal domain with only 91 residues, including 11 half-cystines, that has significant sequence identity with the carboxyl-terminal, disulfide-rich domain of porcine submaxillary mucin (Fig. 7).

REFERENCES
1. Eckhardt, A. E., Timpte, C. S., DeLuca, A. W., and Hill, R. L. (1997) J. Biol. Chem. 272, 33204–33210
2. Gerken, T. A., Owens, C. L., and Pasumarthy, M. (1997) J. Biol. Chem. 272, 9709–9719
3. Sadler, J. E. (1991) J. Biol. Chem. 266, 22777–22780
4. Joba, W., and Hoffmann, W. (1997) J. Biol. Chem. 272, 1805–1810
5. Gum, J. R., Jr., Hicks, J. W., Toribara, N. W., Rothe, E. M., Lagace, R. E., and Kim, Y. S. (1997) J. Biol. Chem. 272, 21375–21383
6. Probst, J. C., Gerken, E.-M., and Hoffmann, W. (1999) Biochemistry 28, 6240–6244
7. Lesuffleur, T., Roche, F., Hill, A. S., Lacasa, M., Fox, M., Swallow, D. M., Zweibaum, A., and Real, F. X. (1995) J. Biol. Chem. 270, 13665–13673
8. Mezzaman, D., Charles, P., Daskal, E., Polyméropoulos, M. H., Martin, B. M., and Rose, M. C. (1994) J. Biol. Chem. 269, 12832–12839
9. Desseyn, J.-L., Aubert, J.-P., van Seuningen, I., Porchet, N., and Laine, A. (1997) J. Biol. Chem. 272, 16873–16883
10. Toribara, N. W., Ho, S. B., Gum, E., Gum, J. R., Jr., Lau, P., and Kim, Y. S. (1997) J. Biol. Chem. 272, 16386–16403
11. Berger, W., Meindl, A., van de Pol, T. J. R., Cremers, F. P. M., Ropers, H. H., Doern, C., Monaco, A., Bergen, A. A. B., Lebo, R., Warburg, M., Zergollern, L., Lorenz, B., Gal, A., Bleeker-Wagemakers, E. M., and Meindl, A. (1992) Nat. Genet. 1, 199–203
12. Chen, Z.-Y., Hendriks, R. W., Johnson, M. A., Powell, J. F., Breakefield, X. O., Sims, K. B., and Craig, I. W. (1992) Nat. Genet. 1, 204–208
13. Meindl, A., Berger, W., Mettinger, T., van de Pol, T. J. R., Achatz, H., Diemer, C., Haasemann, M., Hellebrand, H., Gal, A., Cremers, F. P. M., and Ropers, H. H. (1992) Nat. Genet. 1, 139–143
14. Voorberg, J., Fontijn, R., Calafat, J., Hanssen, H., van Mourik, J. A., and Pannekouk, H. (1991) J. Cell Biol. 113, 185–205
15. Perez-Vilar, J., Eckhardt, A. E., and Hill, R. L. (1996) J. Biol. Chem. 271, 9845–9850
16. Perez-Vilar, J., and Hill, R. L. (1997) J. Biol. Chem. 272, 33410–33415
17. Deng, W. P., and Nickoloff, J. A. (1992) Annu. Rev. Biochem. 60, 81–88
18. Sanchez-Lopez, R., Nicholson, R., and Griswold, M. C., Matrisian, L. M., and Breathnach, R. (1988) J. Biol. Chem. 263, 11892–11899
19. Gunning, P., Leavitt, J., Muscat, G., Ng, S.-Y., and Reddy, L. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4831–4835
20. Braakman, I., Hoever-Litty, H., Wagner, K. R., and Hellenius, A. (1991) J. Cell Biol. 114, 401–411
21. Hebert, D. N., Simon, J. F., Peterson, J. R., and Helenius, A. (1995) Cold Spring Harbor Symp. Quant. Biol. 60, 405–415
22. Schneppenheim, R., Brassard, J., and Ruggeri, Z. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3581–3586
23. Schuback, D. E., Chen, Z. Y., Craig, I. W., Breakefield, X. O., and Sims, K. B. (1995) Hum. Mutat. 5, 285–292
24. Strasberg, P., Alexander, L., Stein, T., Warren, I., Sutherland, J., and Ray, P. N. (1995) Hum. Mol. Genet. 2, 2179–2180
The Carboxyl-terminal 90 Residues of Porcine Submaxillary Mucin Are Sufficient for Forming Disulfide-bonded Dimers
Juan Perez-Vilar and Robert L. Hill

J. Biol. Chem. 1998, 273:6982-6988.
doi: 10.1074/jbc.273.12.6982

Access the most updated version of this article at http://www.jbc.org/content/273/12/6982

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 24 references, 17 of which can be accessed free at http://www.jbc.org/content/273/12/6982.full.html#ref-list-1