C-Mannosylation of MUC5AC and MUC5B Cys subdomains

Juan Perez-Vilar1, Scott H. Randell, and Richard C. Boucher

Cystic Fibrosis/Pulmonary Research and Treatment Center, School of Medicine, CB#7248, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

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We expressed recombinant Cys subdomains in COS-7 cells to examine the role of this highly conserved protein domain in mucin biosynthesis. The entire Cys1 and Cys5 and Cys1 and Cys3 subdomains in MUC5AC and MUC5B, respectively, each with six carboxyl terminal histidine residues, were pulse-labeled with [35S]cysteine/methionine, and the labeled proteins were examined in the culture medium. Under nonreducing conditions, secreted Cys subdomains were monomers, indicating the absence of interchain disulfide bonds. Crosslinking studies suggested the domains are able to interact through very weak noncovalent interactions. Though the domains had apparent Mr consistent with the absence of N- and O-glycans, they could be purified with mannose-specific lectins. Lectin binding was prevented by mutation of the first tryptophan residue in the putative C-mannosylation acceptor motif WXXW, indicating that C-mannosylation is responsible for lectin binding. As judged by pulse-chase experiments, C-mannosylation occurred very early during the domain biosynthesis, likely in the endoplasmic reticulum (ER). Mutation of the WXXW motif or expression of the unmutated domain in CHO-Lec35.1 cells, a C-mannosylation-defective cell line, resulted in reduced secretion of the corresponding Cys subdomains. Live cell imaging of green fluorescent protein fused to the Cys subdomains clearly revealed increased presence of Cys subdomains in the ER of CHO-Lec35.1 cells when compared to the same domains expressed in CHO-K1 cells. Considered together, these studies suggest that the Cys subdomains of MUC5AC and MUC5B are C-mannosylated in their respective WXXW motifs. C-mannosylation is likely required for proper folding of the Cys subdomains and/or for some aspect of ER export during mucin biosynthesis.

Key words: C-mannosylation/mucins/mucin biosynthesis/ MUC5AC/MUC5B

Introduction

MUC5AC and MUC5B are members of the gel-forming mucin subfamily of secreted vertebrate mucins and are thought to be the two major mucins that impart to respiratory mucus its viscoelastic and adhesive properties (Dekker et al., 2002; Gendler and Spicer, 1995; Perez-Vilar and Hill, 1999). These two mucins are secreted by epithelial goblet and the glandular mucous cells into the airways, where they protect the mucosa from infection and chemical damage by binding to inhaled microorganisms and particles that subsequently are removed by the mucociliary system. In lung diseases like cystic fibrosis, the failure to clear mucus, coupled with mucus hypersecretion, decisively contributes to the morbidity and mortality associated with this disease (Quinton, 1999). Despite the importance of mucins in lung defense, the mechanisms of MUC5AC and MUC5B posttranslational processing, assembly, and secretion are poorly understood.

The predominant molecular forms of MUC5AC and MUC5B in mucus are thought to be monomers linked by interchain disulfide bonds to form linear and flexible oligomers/multimers (Hovenberg et al., 1996; Rose et al., 1984; Slayter et al., 1984; Thornton et al., 1990; Wickstrom et al., 1998). However, studies with porcine submaxillary mucin and MUC2 suggest also the existence of branched oligomers/multimers (Godl et al., 2002; Perez-Vilar and Hill, 1999). Each gel-forming mucin monomer consists of a predominant heavily O-glycosylated central region flanked at both sides by different cysteine-rich domains (Figure 1A). Some of the later domains, including D- and CK domains (Perez-Vilar, unpublished data), are involved in the assembly of disulfide-linked oligomers/multimers (Godl et al., 2002; Perez-Vilar and Hill, 1999). The central O-glycosylated regions in MUC5AC (Escande et al., 2001), MUC5B (Desseyn et al., 1997) and its murine counterpart, Mic5b (Escande et al., 2002), are formed by threonine/serine-rich sequences, to which O-glycans are covalently linked, alternating with 110-residue-long, half-cysteine rich regions known as the Cys subdomains. Nine and seven homologous Cys subdomains are found scattered in the central region of MUC5AC (GenBank/EMBL accession numbers AJ298317, AJ298318, AJ2983119 and AJ292079) and MUC5B (GenBank/EMBL accession number Z72496), respectively (Desseyn et al., 1997; Escande et al., 2001) (Figure 1A). Only the Cys subdomains show amino acid homologies between MUC5AC and MUC5B, whereas the Ser/Thr-rich domains have different amino acid sequences. Cys subdomains are present in other vertebrate gel-forming mucins, including MUC2, rMuc2, and porcine gastric mucin (Cao et al., 1999; Perez-Vilar and Hill, 1999). The high intra- and interspecies protein homologies suggest the Cys subdomains play critical but undefined roles in mucus homeostasis.

Mucin Cys-subdomains show significant homologies with a protein domain in human cartilage intermediate layer protein (CILP) (Lorenzo et al., 1998) and especially with a protein domain repeated 13 times in the mucous protein oikosin 1 of...
the larvacean tunicate *Oikopleura dioica* (Spada et al., 2001). Neither the tertiary structure nor the specific functions of these two proteins are known, though it has been proposed that they have structural (rather than regulatory) roles in the organization of the articular cartilage and the larvacean mucous houses, respectively. The presence of mucin Cys subdomain–like protein regions in larvaceans suggests this kind of protein domain evolved from a common ancestor protein present prior to the divergence of urochordates and vertebrates (Spada et al., 2001). From the 10 invariant cysteine residues in the mucin Cys subdomains (Figure 1B), 6 are found conserved in the repeated domain in oikosin 1, of which only 4 are conserved in CLIP. A potential C-mannosylation WXXW acceptor site (Loffler et al., 1996; Desseyn et al., 1997; Escande et al., 2001).

Fig. 1. The Cys subdomains in MUC5B and MUC5AC. (A) Schematic representation of the major domains found in the polypeptide chains of MUC5AC and MUC5B as deduced from their cDNA sequences. Yellow boxes indicate O-glycosylated regions. D, D domains; M, MUC11p15-type domains; CS, Cys subdomains; C, C domains; CK, CK domains. (B) Complete amino acid sequences of MUC5AC/Cys5 and MUC5B/Cys3, the two Cys subdomains most thoroughly studied in the present work. The positions of identical or chemically related amino acids are indicated by asterisks and dots, respectively. The potential C-mannosylation acceptor motifs WXXW are underscored. (C) Alignment of the amino acid sequences comprising the potential C-mannosylated motif WXXW in the Cys subdomains of MUC5AC and MUC5B (Desseyn et al., 1997; Escande et al., 2001).
Vliegenthart and Casset, 1998) is found in 11 of the 13 repeats in oikosin 1 and at the N-terminal side of the Cys subdomains of MUC5AC and MUC5B (Figure 1C). The less common variant WXXF/Y is present in the other two oikosin repeats (Spada et al., 2001), and a WXXL sequence is at the same position in CILP (Lorenzo et al., 1998). Whether or not these potential C-mannosylated acceptor motifs are glycosylated has not yet been determined. C-mannosylation differs from O- and N-glycosylation in that it involves a covalent attachment of a single α-mannose residue to the indole C2 carbon atom of the first Trp in WXXW peptide motifs (Hofsteenge et al., 1994). C-mannosylation is found among secreted and membrane proteins, although its function still undetermined.

Our early biochemical studies with recombinant mucin domains expressed in COS-7 cells have identified structural features that could play critical roles in the processing, assembly, and secretion of vertebrate gel-forming mucins (Perez-Vilar et al., 1996, 1998; Perez-Vilar and Hill, 1998a,b, 1999). This model system circumvents some of the difficulties associated with the high molecular weight, polymorphism, and extent of glycosylation of these glycoproteins for studying the function of unique mucin domains. Using this model system and also expression in Chinese hamster ovary (CHO) cells to explore mucin Cys subdomain function during mucin biosynthesis, we show here that MUC5AC and MUC5B Cys subdomains are C-mannosylated and that lack of C-mannosylation results in the arrest of these protein domains in the endoplasmic reticulum (ER).

Results

Expression and secretion of mucin Cys subdomains

COS-7 cells were transfected with expression plasmids encoding fusion proteins containing the signal peptide of murine Igμ-chain followed by MUC5AC/Cys1, MUC5AC/Cys5, MUC5B/Cys1, or MUC5B/Cys2 subdomains with six consecutive histidine residues at their carboxyl termini. Control cells were transfected with the parental vector, pSecTag2A, lacking any mucin-coding sequence. Twenty-four hours after transfection, the cells were incubated for 4 h with [35S]-labeled cysteine/methionine and the mucin Cys subdomains isolated from the medium by adsorption to and elution from a metal-affinity adsorbant. The eluates were reduced with 2-mercaptoethanol and analyzed by sodium dodecyl sulfate (SDS)–gel electrophoresis and autoradiography (Figure 2A). Although no specific proteins were detected in the media of cells transfected with the parental plasmid (lane 1), single protein bands were detected in the medium of cells transfected with expression plasmids for MUC5AC/Cys1 (lane 2), MUC5AC/Cys5 (lane 3), or MUC5B/Cys1 (lane 4). As shown in Figure 2B, the apparent molecular weights of all mucin Cys subdomains tested were in agreement with the expected Mr, deduced from their respective cDNAs (Desseyn et al., 1997; Escande et al., 2001). Figure 2C shows that MUC5B/Cys2 was detected in the medium as two proteins. The predominant protein had a molecular weight of around 14,000, which is consistent with the expected Mr, ~20,000, was judged to correspond to an N-glycosylated species based on its sensitivity to the presence of tunicamycin, an inhibitor of protein N-glycosylation, which only allowed detection of the smaller but predominant species (not shown). Among the mucin Cys subdomains, only MUC5B/Cys2 has an Asn-X-Ser/Thr peptide motif; therefore it was not surprising that only this Cys subdomain was secreted from COS-7 cells as a mixture of unglycosylated and N-glycosylated species. These results validate the specificity of the metal-affinity adsorbant for the isolation of the mucin Cys subdomains and show that these domains, with the exception of MUC5B/Cys2, are secreted without extensive posttranslational modifications. In similar experiments, MUC5B/Cys3 was expressed in the medium at a levels comparable to MUC5AC/Cys5, therefore plasmids expressing these two protein domains were used for the majority of the studies described.

The mucin Cys subdomain does not form interchain disulfide-linked species

To assess whether disulfide-bonded species of mucin Cys subdomains were formed, the subdomains in the culture
Cys subdomains, only monomers of the Cys subdomains that this domain is rich in intrachain disulfide bonds. These medium of transfected COS-7 cells were purified and analyzed by gel electrophoresis and autoradiography. EDC/S-NHS and analyzed by reducing SDS–gel electrophoresis and autoradiography. EDC/S-NHS cross-links proteins through a different mechanism, which involves creation of free amino groups from carboxyl groups.

Detection of unreduced mucin Cys subdomains in SDS gels. COS-7 cells were transfected with pSecTag2A (lanes 1 and 2), pMUC5AC/Cys1 (lanes 3 and 4), pMUC5AC/Cys5 (lanes 5 and 6), or pMUC5B/Cys3 (lanes 7 and 8) and incubated 24 h later in [³⁵S]-amino acids. The His-tagged proteins were purified from the culture medium with TALON-IMAC beads. Proteins were analyzed by SDS–gel electrophoresis and autoradiography with (lanes 1, 3, 5, and 7) or without (lanes 2, 4, 6, and 8) previous reduction with 2-mercaptoethanol (MET). The molecular weights of the standards (mw) are in thousands.

Figure 3. Detection of unreduced mucin Cys subdomains in SDS gels. COS-7 cells were transfected with pSecTag2A (lanes 1 and 2), pMUC5AC/Cys1 (lanes 3 and 4), pMUC5AC/Cys5 (lanes 5 and 6), or pMUC5B/Cys3 (lanes 7 and 8) and incubated 24 h later in [³⁵S]-amino acids. The His-tagged proteins were purified from the culture medium with TALON-IMAC beads. Proteins were analyzed by SDS–gel electrophoresis and autoradiography. EDC/S-NHS and analyzed by reducing SDS–gel electrophoresis and autoradiography. EDC/S-NHS cross-links proteins through a different mechanism, which involves creation of free amino groups from carboxyl groups.

Detection of noncovalent species of the mucin Cys subdomain

It has been suggested that protein–protein interactions among nonglycosylated protein domains in mucins could be the basis for formation of noncovalent linear and/or branched structures by which covalent mucin multimers are held together (Cao et al., 1999). Considering the locations of the Cys subdomains in the mucin polypeptides, interdispersed among heavily O-glycosylated regions, they are potential candidates for mediating such noncovalent interchain bonds. Thus cross-linking agents, including glutaraldehyde and bis(sulfosuccinimidyl)suberate (BS3), were used to test for formation of noncovalent species of the mucin Cys subdomains. Because these cross-linkers react preferentially with primary amines, MUC5AC/Cys5 and MUC5B/Cys3, which have four and five lysine residues, respectively, were used for these experiments. Figure 4A shows mucin MUC5B/Cys3 purified from the culture medium of transfected cells, reacted with increasing concentrations of glutaraldehyde, and analyzed by nonreducing gel electrophoresis. Normal exposure times of the gel showed just monomeric species (not shown), though cross-linked monomers migrated as very broad bands, and it was also evident in gels exposed for longer periods of time (compare lane 1 with lanes 2–4), likely reflecting modification of amino acid side chains by glutaraldehyde. In the longer-exposure gels, however, protein bands corresponding to dimeric and trimeric species of MUC5B/Cys3 were observed when proteins were previously incubated with glutaraldehyde (lanes 2–4). The relative amounts of MUC5B/Cys3 dimers/oligomers were similar irrespective of glutaraldehyde concentration (compare lanes 2 and 3) or the reaction time (compare lanes 3 and 4), suggesting that the intermolecular noncovalent interaction was very weak. Similar results were obtained when the purified MUC5B/Cys3 was cross-linked with the homobifunctional cross-linker BS3 or when MUC5B/Cys3 was cross-linked with BS3 prior purification, that is, in the culture medium, except that Cys subdomain monomers did not migrate as the broad bands observed with glutaraldehyde (data not shown).

To determine if the extent of noncovalent dimerization/oligomerization of a given Cys subdomain was affected by the presence of a different mucin Cys subdomain, COS-7 cells were cotransfected with pMUC5AC/Cys5 and either one of the plasmids MUC5AC/Cys1, MUC5B/Cys1, or MUC5B/Cys3. These studies should test for the existence of either cooperative effects or the occurrence of strong heterotypic interactions. The proteins secreted into the medium were purified, cross-linked with BS3 or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimidehydrochloride(EDC)/N-hydroxysuccinimide (S-NHS) and analyzed by reducing SDS–gel electrophoresis and autoradiography. EDC/S-NHS cross-links proteins through a different mechanism, which involves creation of free amino groups from carboxyl groups in proteins. In our cotransfection protocols, more than 70% of the cells, as assessed by coexpression of fluorescent proteins, are cotransfected (not shown; see also Perez-Vilar and Hill, 1997).

As shown in Figure 4B, and consistent with studies shown in Figure 4A, monomers of the mucin Cys subdomains were the major species observed. MUC5AC/Cys5 monomers could be readily distinguished by SDS–gel electrophoresis because the molecular weight of MUC5AC/Cys5 is higher than the molecular weight of MUC5AC/Cys1, MUC5B/Cys1, or MUC5B/Cys3 (see bottom insert in Figure 4B and also Figure 2B). In the same gels and in spite of the cross-linker employed, protein bands corresponding to dimers/oligomers of the Cys subdomains were barely detected and needed longer exposure times to be clearly identified, as shown in the full gel at the top of Figure 4B. Monomeric and dimeric but not oligomeric species of ECD/S-NHS-treated proteins (lanes 2, 4 and 6), migrated slightly slower in SDS gels than those cross-linked with BS3.
Fig. 4. Detection of noncovalent dimers and oligomers of the mucin Cys subdomains. [35S]-labeled MUC5B/Cys3 expressed in and purified from the culture media of COS-7 cells were utilized in gel electrophoresis as in Figure 2 and then incubated for the indicated period of time with PBS in the absence (lane 1) or presence of 0.01% (v/v) (lane 2) or 0.1% (v/v) (lanes 3 and 4) of glutaraldehyde (Glut). Proteins were then analyzed by nonreducing SDS–gel electrophoresis. M, D, and T indicate the positions of monomers, dimers, and trimers of the corresponding Cys subdomain, respectively, in all but lane 1, which only shows monomers. The molecular weights of the standards (mw) are in thousands. COS-7 cells were cotransfected with pMUC5AC/Cys5 and either of the vectors pMUC5AC/Cys1 (lanes 1 and 2), pMUC5B/Cys1 (lanes 3 and 4) or pMUC5B/Cys3 (lanes 5 and 6) at a plasmid ratio (w/w) of 1:10, 1:10, or 1:2, respectively. After 24 h, cells were metabolically labeled with [35S]-amino acids and His-tagged proteins purified from the culture medium with TALON IMAC resin and cross-linked with BS3 (lanes 1, 3, and 5), or with 1 mM each of EDC/S–NHS (lanes 2, 4, and 6) for 30 min at 25°C. Proteins were then analyzed by reducing SDS–gel electrophoresis and fluorography. Insert in the bottom is a detail of the same gel, which was exposed to the radiographic film for a shorter period of time, showing the positions of monomers, dimers, and trimers of the corresponding Cys subdomain, respectively, in all lanes. A dot indicates position of the trimeric species in lane 1.

Considered together, the cross-linking studies are inconsistent with the existence of strong, noncovalent, homo- or heterotypic interactions between Cys subdomains at the conditions tested.

C-Mannosylation of mucin Cys subdomains

The presence of a WXXW amino acid sequence in the N-terminal side of all mucin Cys subdomains is one of their most distinctive features (Desseyn et al., 1997; Escande et al., 2001) (Figures 1B and 1C). Because it has been recently shown that conserved WXXW sequences in extracellular proteins are acceptor motifs for protein C-mannosylation, by which a single mannose is bound to the first tryptophan in the motif (Krieg et al., 1997), we initiated studies to test whether mucin Cys subdomains were C-mannosylated. The glycosyltransferase activity responsible for C-mannosylation has been detected in COS-7 cells (Krieg et al., 1997); therefore this cell system was suitable for our purposes. However, attempts to detect mannosylation of MUC5AC/Cys5 or MUC5B/Cys3 by metabolic labeling of the protein using [3H]-mannose or [3H]-glucose were unsuccessful (data not shown). The fact that only a single [3H]-mannose can be added per mucin Cys subdomain, together with the low energy of the [3H]-mannose, the competition for this monosaccharide by other glycosylation processes, and the rather low level of protein expression obtained in our experimental system, provide a likely explanation for the lack of sensitivity of this approach.

As an alternative strategy, we used affinity chromatography with mannose-specific lectins as adsorbents of MUC5AC/Cys5 and MUC5B/Cys3. This method, though indirect and not of general application, seemed especially suitable for our case for two reasons. First, MUC5AC/Cys5 and MUC5B/Cys3 are not N-glycosylated and, based on their molecular weights, lack large carbohydrate chains. N-linked oligosaccharide chains are the major source of mannose, whereas O-linked oligosaccharide or glycosaminoglycan chains might interfere nonspecifically with the lectin-binding assays. Second, a lectin-based approach permitted analysis of low amounts of radioactive mucin domains. Galanthus nivalis agglutinin (GNA) (Hester and Wright, 1996) andLens culinaris agglutinin (LCA) (Loris et al., 1994), two structurally unrelated lectins that recognize nonreducing terminal mannose residues, were used for these studies.

Figure 5A shows that MUC5AC/Cys5 can be purified by lectin absorption using GNA–agarose beads. In these studies, COS-7 cells expressing MUC5AC/Cys5 were incubated with [35S]-amino acids and secreted proteins purified by different means and analyzed by gel electrophoresis. Metal-affinity absorption of the secreted proteins yielded one protein band of Mr ~ 13,100 (lane 1) corresponding to MUC5AC/Cys5, which is consistent with results shown in Figure 2A. Direct incubation of the culture medium with GNA–agarose beads, however, showed the binding of many glycoproteins to the adsorbent, of which one with a Mr ~ 13,100 was among the most abundant (lane 2). This protein band was not observed following lectin-affinity purification of culture media from cells transfected with the parental plasmid, pSecTag2A (data not shown).
The 13,100 \( M_r \) protein could be repurified by metal-affinity absorption after it was eluted from the GNA-agarose resin with buffered 4 M guanidine-HCl (lane 3), which identified this protein as the His-tagged MUC5AC/Cys5. Consistent with these results, MUC5AC/Cys5 bound to and eluted from the metal-affinity adsorbant, could be repurified with GNA-agarose (lane 4). Binding to GNA-agarose was judged to be mannose-dependant based on the following observations: (1) the mucin Cys subdomains did not bind to uncoupled agarose (data not shown); (2) mucin Cys subdomains could be purified using another, structurally unrelated, mannose-specific lectin (LCA) (Furmanek and Hofsteenge, 2000) (Figure 5B); and (3) binding of the Cys subdomain to the lectin adsorbant was substantially reduced in the presence of free mannose in the binding buffer (Figure 5C). These results suggest mucin Cys subdomains were mannosylated during their biosynthesis in transfected cells.

The absence of N-glycosylation recognition sites in MUC5AC/Cys5 (Escande et al., 2001), its apparent molecular weight (Figure 2B), and the lack of mannose residues in O-glycans, made it unlikely that N- or O-linked oligosaccharide chains were responsible for lectin binding. C-mannosylation, however, which would contribute 162 Da to the mass of MUC5AC/Cys5, seemed the best candidate modification to explain the binding of the Cys subdomain to GNA or LCA lectin. To provide further evidence supporting this conclusion, site-specific mutagenesis was used to test whether lectin binding was dependent on the C-mannosylation acceptor motif in the mucin Cys subdomains. The first (not the second) tryptophan residue in the WXXW motif-encoding sequence of MUC5AC/Cys5 (Figure 1C) was changed to alanine for two reasons. First, this is the tryptophan residue to which mannose is usually covalently bound in C-mannosylated proteins (Krieg et al., 1997). Second, Rose et al. (1989) identified in tryptic digests of airway mucins the peptide WFDVDFSPGPYGGGKE TYN, which the first residue corresponds to the second tryptophan residue in WXXW motif-encoding sequence of MUC5AC/Cys5 (Figure 1C) was changed to alanine for two reasons. First, this is the tryptophan residue to which mannose is usually covalently bound in C-mannosylated proteins (Krieg et al., 1997). Second, Rose et al. (1989) identified in tryptic digests of airway mucins the peptide WFDVDFSPGPYGGGKE TYN, which in the first residue corresponds to the second tryptophan residue in WXXW motif-encoding sequence of MUC5AC/Cys5 (Figure 1C) was changed to alanine for two reasons. First, this is the tryptophan residue to which mannose is usually covalently bound in C-mannosylated proteins (Krieg et al., 1997). Second, Rose et al. 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SDS gels slightly faster than MUC5AC/Cys5, consistent with a reduction in its mass. Indeed, the replacement of one Trp residue with an Ala residue and the eventual abrogation of C-mannosylation would reduce the molecular mass of the mutated Cys subdomain by 279 Da. No protein bands were detected after elution of MUC5AC/Cys5(W/A) from the metal-affinity adsorbant and subsequent absorption of the mutated domain to GNA-agarose beads (lane 2), in clear contrast with the results described in Figure 5A for the unmutated Cys subdomain. These results indicated that binding of MUC5AC/Cys5 to mannose-specific lectins depended on the C-mannosylation recognition motif WXXW, which strongly suggests the mucin Cys subdomains are C-mannosylated.

C-mannosylation of the mucin Cys subdomains is an early modification

COS-7 pulse-chase studies were performed to determine the subcellular compartment where mannosylation occurred. Figure 7 shows the gel electrophoretic patterns of the cellular proteins from COS-7 cells transfected with MUC5AC/Cys5 when pulsed for 5 min with [35S]-amino acids and then chased with unlabeled medium over time. In these studies, proteins were first purified from cell lysates with metal-affinity adsorbant and then eluted with unlabelled medium. No protein bands were detected after elution of MUC5AC/Cys5(W/A) from the metal-affinity adsorbant. Figure 7 shows the gel electrophoretic patterns of the cellular proteins from COS-7 cells transfected with MUC5AC/Cys5 when pulsed for 5 min with [35S]-amino acids and then chased with unlabeled medium over time. In these studies, proteins were first purified from cell lysates with metal-affinity resin, eluted in buffered ethylenediamine tetra-acetic acid (EDTA), and repurified with GNA-agarose beads. Even at 0 min of chase, the MUC5AC/Cys5 domain binds to the mannose specific-lectin (lane 1). Addition of brefeldin A, which disorganizes the Golgi complex (Jackson, 2000), did not prevent lectin binding (lane 2).

As expected, following 10 min in unlabeled medium, MUC5AC/Cys5 purified from control (lane 3) or brefeldin A–treated cells (lane 4) bound to the lectin. Although it may appear that the latter band migrated differently than the others, this was caused by a small crack in the gel shown and not by differences in the electrophoretic mobilities of the corresponding proteins. These results suggest that C-mannosylation of the mucin Cys subdomains takes place very rapidly during the biosynthesis of these proteins. These observations are consistent with previous studies by others showing that C-mannosylation requires dolichol-P-mannose (Doucey et al., 1998), which is synthesized in the ER.

Unmannosylated mucin Cys subdomains are poorly secreted

Figure 8A shows the results from experiments in which COS-7 cells were transfected with three independent clones of plasmid pMUC5AC/Cys5 (lanes 1–3) or pMUC5AC/Cys5(W/A) (lanes 4–6), respectively, incubated for 24 h, and metabolically labeled with [35S]-amino acids. Secreted proteins were purified with IMAC beads and analyzed by SDS–gel electrophoresis and fluorography. (B) CHO-Lec35.1 cells were transfected with pSecTag2A (lanes 1–3) or pMUC5AC/Cys5 (lanes 4–6) and 24 h later metabolically labeled with [35S]-amino acids for 15 min and chased in unlabeled culture media for 4 h. At the indicated chase time, proteins were purified from Triton-X100 cell lysates (lanes 1, 2, 4, and 5) or the culture medium (lanes 3 and 6) with TALON IMAC beads and analyzed by nonreducing SDS–gel electrophoresis and fluorography. Molecular weights of the standards (mw) are in thousands.
translational efficiencies between the parental and mutated sequences because both types of constructs yield similar protein amounts in coupled *in vitro* transcription/translation assays (data not shown). These results suggest that the Cys subdomains lacking the C-mannosylation acceptor motif are poorly secreted.

To avoid the uncertainty associated with the use of mutant versions of the Cys subdomain in COS-7 cells, especially considering the tight relationship between protein folding and secretion rate found in many secreted/membrane proteins, we expressed MUC5AC/Cys5 in CHO-Lec35.1 cells. It has been demonstrated that these cells accumulate but do not use dolichol-P-mannose and, therefore, are defective in protein C-mannosylation (Anand et al., 2001). The parental cell line, CHO-K1, displays normal levels of protein C-mannosylation (Anand et al., 2001) and, accordingly, shows normal secretion of the Cys subdomain when transiently transfected with pMUC5AC/Cys5, as for COS-7 cells (not shown). However, as shown in Figure 8C, the intracellular pool of MUC5AC/Cys5 synthesized in CHO-Lec35.1 cells during a 15-min pulse with 35S-s-aminos acids (lane 4) was not reduced after 4 h of chase in unlabeled medium (lane 5), and only traces of the domain were detected in the culture medium after the chase (lane 6), as for the AXW mutant of the same domain expressed in COS7 cells. These results show that MUC5AC/Cys5 is largely arrested inside C-mannosylation-defective cells soon after it is synthesized. These and the studies with AXW mutants in COS-7 cells strongly suggest that the lack of C-mannosylation is responsible for intracellular retention of the mucin Cys subdomains.

To determine the intracellular fate of the nonmannosylated Cys subdomains, the Cys subdomains were fused to the C-terminal end of green fluorescent protein (GFP), itself fused to the C-terminal end of a signal peptide, and the trafficking of the corresponding proteins assessed by GFP methods in live CHO-K1 or CHO-Lec35.1 cells. Preliminary experiments indicated that GFP-mucin fusion proteins were secreted from COS-7 and CHO-K1 cells but in lesser amounts from CHO-Lec35.1 cells, showing that GFP did not alter the secretion pattern of the mucin Cys subdomains. Figure 9A shows confocal images of live cells 24 h after transfection with plasmids encoding secreted GFP, named SHGFP (a and d) or the GFP-fusion proteins SHGFP-MUC5AC/Cys5 (b and e) and SHGFP-MUC5B/Cys3 (c and f), respectively. In CHO-K1 (a) and CHO-Lec35.1 cells (d), SHGFP was predominantly found in perinuclear tubulovesicular structures characteristic of the Golgi complex, very likely representing proteins in their way to secretion. These results show that the intracellular trafficking of SHGFP is not affected by the phenotype, including the lack of protein C-mannosylation, of CHO-Lec35.1 cells. This is not unexpected considering that in nature native GFP is not a secreted protein and, accordingly, does not have N-linked oligosaccharides or C-mannoses.

When MUC5AC/Cys5 or MUC5B/Cys3 were fused to GFP, the resulting proteins, named SHGFP-MUC5AC/Cys5 and SHGFP-MUC5B/Cys3, respectively, showed an intracellular distribution different than SHGFP, irrespective of the cell type under study. Thus in CHO-K1 cells, SHGFP-MUC5AC/Cys5 (Figure 9A, b) and SHGFP-MUC5B/Cys3 (c), were found in the Golgi complex perinuclear region and, in addition, in an intricate reticulum extending off the nuclear envelope, which is characteristic of the ER. In CHO-Lec35.1 cells, however, both of these proteins were largely predominant in the ER (e and f, respectively). As shown in Figure 9B, SHGFP-MUC5AC/Cys5 colocalized in live CHO-Lec35.1 cells with an enhanced cyan fluorescent protein (ECFP) fused to two ER sorting sequences (see Material and methods for details) (see right cell in a, b and c), which confirms the intricate reticulum, where the Cys subdomain is accumulated, indeed represented ER. These results showed that in C-mannosylation-defective cells, the Cys subdomains are largely confined to the ER and, ultimately, poorly secreted.

**Discussion**

Though the Cys subdomains exhibit the highest DNA and protein homologies among gel-forming mucins, their biochemical and functional properties are unknown. Our analysis of the recombinant Cys subdomains secreted by transfected cells demonstrated absence of interchain disulfide bound species (Figure 3). This result predicts the large, central O-glycosylated regions of MUC5AC and MUC5B, where the Cys-subdomains are found (Figure 1A), are not covalently connected to themselves, consistent with early morphological studies (Rose et al., 1984; Slayter et al., 1984; Thornton et al., 1990). In consequence, formation of covalent oligomers/multimers of gel-forming mucins can be explained by interchain disulfide bonding of the N-terminal D-domains and the C-terminal CK domains, respectively, as proposed earlier (Perez-Vilar and Hill, 1999).

Rheological studies suggest mucus is formed by a mucin network held together by weak noncovalent bonds with the likely intervention of certain ions (Bansil et al., 1995; Madsen et al., 1998; Raynal et al., 2003), although other studies support the view that mucus viscoelastic properties are determined primarily by entanglement of the mucin oligomers/multimers (Carlstedt et al., 1985; Verdugo, 1990). Moreover, early reports documented the tendency of human tracheobronchial mucins, likely a mixture of MUC5AC and MUC5B, to form aggregates at low salt concentrations (Rose et al., 1984; Slayter et al., 1984), and recent investigations suggest that aggregation of respiratory mucins is caused by interchain hydrophobic interactions (Bromberg and Barr, 2000). Furthermore, noncovalent interactions are thought to be critical during the packing of mucin precursors inside mucus granules of goblet/mucous cells. The specific roles that the different protein domains in mucins play in these interactions have not been defined yet. Interestingly, the Cys subdomains in porcine gastric mucin have been proposed to mediate hydrophobic protein-protein interactions critical for mucus gel formation at low pH (Cao et al., 1999). Our cross-linking studies, however, argued against the existence of strong noncovalent homo- and heterotypic interactions among the mucin Cys subdomains at neutral or slightly acidic pH, and only very weak noncovalent interactions were revealed using cross-linkers (Figure 4). The biological significance (if any)
of these very weak interactions is not clear at present. Unfortunately, the amounts of expressed Cys subdomains in our cell system are unsuitable for using alternative, more powerful and precise binding techniques. In any case, it is tempting to speculate that in vivo the Cys subdomains might be protein regions where disulfide-linked oligomers/multimers of MUC5AC or MUC5B contact one another, especially when certain conditions, for example, regarding pH and mucin concentration, are met.

MUC5AC and MUC5B, like others, are highly glycosylated with N- and especially O-linked oligosaccharides. Glycosylation is fundamental to their ability to form the mucus gels, which underlie the mucociliary clearance mechanism, and to interact with many different compounds. The present studies suggest that a third type of glycosylation, C-mannosylation, is also present in MUC5AC and MUC5B. Thus mucin Cys subdomains are secreted from transiently transfected cells as C-mannosylated proteins, based on the observations that MUC5AC/Cys5 and MUC5B/Cys3 bind to the mannose-specific lectins, GNA and LCA (Figure 5) and that this binding depends on the C-mannosylation recognition sequence WXXW in these domains (Figure 6). The absence of N-glycosylation recognition sites in MUC5AC/Cys5 and MUC5B/Cys3, eliminating the major source of mannose residues in mammalian glycoproteins, made feasible the use of mannose-specific lectins to reveal C-mannosylation of this domain. Moreover, the apparent molecular weights

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Fig. 9. Intracellular distribution of mucin Cys subdomains in live CHO-K1 and CHO-Lec35.1 cells. (A) CHO-K1 (a, b, and c) or CHO-Lec35.1 cells (d, e, and f) were transfected with plasmids expressing SHGFP (a and d), SHGFP-MUC5AC/Cys5 (b and e) or SHGFP-MUC5B/Cys3 (c and f). After 24 h, live cells were visualized with a laser scanning microscope at 37°C on the microscope stage using a 63 × 1.4 NA objective. Shown are XY projections made from 20 confocal images. (B) CHO-Lec35.1.2 cells were cotransfected with plasmids pSHGFP-MUC5AC/Cys5 and pECFP-ER and 24 h later visualized with a laser scanning microscope at 37°C. Confocal images of SHGFP-MUC5AC/Cys5 (a), ECFP-ER (b), and both (c) in live cells were taken using 488 nm laser excitation for GFP and 413 nm for ECFP and analyzed using Zeiss LSM software. Yellowish signal in c indicates colocalization of both proteins. One (1) indicates a cell expressing ECFP-ER and SHGFP-MUC5AC/Cys5; two (2) indicates a cell expressing just ECFP-ER.
of these Cys subdomains were consistent with unmodified or under glycosylated proteins (Figure 2B), excluding the possibility of nonspecific interactions between the GNA or LCA lectins and O-linked oligosaccharide or glycosaminoglycan chains. Furthermore, other forms of protein glycosylation that add single monosaccharides (Spiro, 2002) and therefore could interfere with our lectin assay, seemed highly unlikely to occur in the mucin Cys subdomains. Thus N-acetyl-glucosamine-Ser/Thr linkages are not present in secretory proteins and both fucose-Ser/Thr and glucose-Ser linkages are primarily found in epidermal growth factor-like domains (Spiro, 2002), which are protein domains structurally unrelated to the mucin Cys subdomains (Deseyn et al., 1997; Escande et al., 2001).

The change of the WXXW motif in MUC5AC/Cys5 to an AXXW sequence prevented binding of the domain to either LCA or GNA (Figure 6). It has been demonstrated that WXXW motifs in C-mannosylated proteins are the major acceptor sequences for the glycosyltransferase involved in this kind of glycosylation (Krieg et al., 1998; Doucey et al., 1999). Therefore the binding of GNA or LCA to the Cys subdomains can be explained by the fact that this protein domain is C-mannosylated in its WXXW motif. This conclusion is supported by studies suggesting the primary (rather than tertiary) structure determines whether a protein is C-mannosylated. Thus the sequence TWANWFTET in RNase2 has all the information required for C-mannosylation of this protein (Doucey et al., 1998). The corresponding sequence in MUC5AC/Cys5, TWTTWFDV (Figure 1B and 1C), is very similar, strongly suggesting that the same structural information that determines C-mannosylation of RNase2 is conserved in MUC5AC/Cys5 and likely in all mucin Cys subdomains (Figure 1C). Considered together, these observations suggest that the mucin Cys subdomains in MUC5AC and MUC5B are C-mannosylated in their respective WXXW motifs. The question arises whether C-mannosylation occurs in airway goblet/mucous cells. Unfortunately, neither the enzyme responsible for this modification nor its gene has been characterized; therefore we are unable to confirm its presence in particular cell types. However, the current evidence strongly suggest C-mannosylation, like O- and N-glycosylation, is a widespread modification that has been found in all tissue and animal species tested (Furmanek and Hofsteenge, 2000), including the lung (Hofsteenge, personal communication).

Our attempts to demonstrate or deny C-mannosylation in native MUC5AC and MUC5B have failed. Thus in these studies, which involve the generation, fractionation, and direct mass spectrometry sequencing of tryptic peptides derived from purified airways MUC5AC/MUC5B mucins, we have identified a set of peptides representative of many of the protein regions in the mucin Cys subdomains. Peptides located at the N-terminal side of the mucin Cys subdomains, where the WXXW motifs are located, were not found (Kesimer et al., unpublished data). The proximity of O-glycosylated, Ser/Thr-rich regions at the N-terminal side of the Cys subdomains, and therefore close to their respective N-terminal WXXW motifs, may have prevented the isolation or identification of these peptides, although alternative strategies are in progress. In any case, so far these results suggest native Cys subdomains, like their recombinant counterparts (Figure 2), are not modified with N- and O-linked oligosaccharides, glycosaminoglycan chains, or other major modification, supporting the view the recombinant Cys subdomains expressed in COS-7/CHO cells are valid model systems of their respective native domains.

The glycosyltransferase responsible for C-mannosylation has not yet been characterized, but it seems to be located in microsomal fractions and requires dolichol-P-mannose as donor of mannose (Anand et al., 2001; Doucey et al., 1998), a compound synthesized in the ER, which suggests this organelle is the intracellular site for protein C-mannosylation. Our pulse-chase studies showed that C-mannosylation of MUC5AC/Cys5 occurred very rapidly and was not affected by the presence of brefeldin A (Figure 7), a compound that disorganizes the Golgi complex. These data are consistent with the conclusion that protein C-mannosylation takes place in the ER. Moreover, as will be discussed, lack of C-mannosylation prevents transport of the Cys subdomains out of the ER. Therefore C-mannosylation, together with N-glycosylation and covalent dimerization via the CK domains (Perez-Vilar and Hill, 1999; Asker et al., 1998a,b; Perez-Vilar unpublished data), would be the three major modifications of the MUC5AC and MUC5B polypeptides in the ER.

Although the role of protein C-mannosylation is unclear at present, our studies clearly suggest this modification is required for proper transport of the Cys subdomain out of the ER. Thus Cys subdomains with mutated WXXW motifs were poorly secreted from COS-7 cells (Figure 8A), whereas their normal counterparts were arrested inside the ER only when expressed in C-mannosylation defective cells (Figures 8B and 9A). It is well documented that misfolded or partially folded proteins are retained in the ER by the quality control mechanism that operates in this organelle (Ellgaard and Helenius, 2003). Hence, a critical role of C-mannosylation during mucin Cys subdomain folding would explain that in its absence the domains are retained inside the ER. There are three lines of indirect evidence supporting such a role for this modification. First, structural studies with recombinant eosinophil-derived neurotoxin, a C-mannosylated RNase, indicated C-mannosylation stabilizes its N-terminal loop, likely by keeping the first tryptophan in the WXXW motif in a specific orientation (Loffler et al., 1996; Vliegenthart and Casset, 1998). Both features are lost in the unglycosylated counterpart of this protein. Second, mutation of the conserved WXXW motif in the erythropoietin receptor arrests the protein in the ER (Hilton et al., 1996), although whether the receptor was C-mannosylated was not studied. Third, experiments with RNase2 suggest C-mannosylation occurs prior to complete protein folding (Krieg et al., 1998), suggesting this modification is cotranslational and hence able to influence folding of the nascent polypeptide. It is tempting to speculate that C-mannosylation of the Cys subdomains together with N-glycosylation of the D, C, and CK domains, cooperate with complementary mechanisms to allow correct folding of MUC5AC and MUC5B in the ER.

Alternatively, C-mannosylation might be part of a mechanism that regulates mucin ER export once mucin precursors are folded and ready to exit the organelle. It is
increasingly clear that although some proteins seem to exit the ER by a bulk flow pathway, others are packaged into transport vesicles (Glick, 2001; Gorelick and Shugrue, 2001). This packaging requires interactions between proteins in transit and vesicular coat subunits and/or cargo receptors. Thus C-mannoses might be involved in lectin-type interactions critical for mucin packaging at ER exit sites. In the ER, mucin precursors are very likely not O-glycosylated, a modification that largely occurs in the Golgi complex (Perez-Vilar and Hill, 1999); therefore the Cys subdomains and their C-mannoses would be accessible to interact with ER proteins.

In conclusion, our studies suggest that a third type of protein glycosylation, C-mannosylation, exists in MUC5AC and MUC5B. C-mannoses would be located in the WXXW motifs at the N-terminal side of the Cys subdomains, which are interdispersed among the O-glycosylated regions of these mucins. C-mannosylation seems to be required during the early stages of mucin biosynthesis, either for the folding of the domain or for some aspect of the transport out of the ER.

Materials and methods

Cloning of MUC5AC and MUC5B Cys subdomains and construction of expression vectors

All the DNA encoding the Cys subdomains studied here were obtained by polymerase chain reaction of human genomic DNA (a gift from Dr. M. Zariwala, CF Center, University of North Carolina) using the following oligonucleotide pairs (MWG Biotech., Greensboro, NC) based on the published sequences of MUC5AC and MUC5B (Desseyn et al., 1997; Escande et al., 2001): MUC5AC/Cys1, 5’-GGCGGGCTGTGGGGAAAGATTGCTCTGTGCTCGCC-3’ and 5’-GGATTTATGTATGATGTATGTGTAGTGAGCGGTCAAGGGCAGGACTCAGTACCTGGACAA-3’ and 5’-GGTTAATGTATGATGTATGTATGTATGTGGAGGGGACATGAC-3’; MUC5B/Cys1, 5’-GGCGGGCTGTGGGGAAAGATTGCTCTGTGCTCGCC-3’ and 5’-GGATTTATGTATGATGTATGTATGTATGTCAG-3’ and 5’-GGTTAATGTATGATGTATGTATGTATGTGGAGGGGACATGAC-3’. These oligonucleotides added a 5’ Ascl restriction site and a 3’ histidine tag-encoding sequence with six consecutive histidine codons before the stop codon, respectively. The amplified DNA sequences of pMUC5AC/Cys1 and pMUC5B/Cys1 were 5’-CCCCGGTGCACCGCGACAACGTGGTTCG-3’ and 5’-CACCCGAAATGTTGAGGGGACAGTGTGTTTGCTGAGCG-3’, respectively. The complete DNA sequences encoding the respective mucin Cys subdomains were verified by DNA sequencing. The transcriptional/translational efficiencies of these two plasmids were assessed by coupled in vitro transcription/translation assays using the T7 TNT system from Promega (Madison, WI) as directed by the manufacturer. 35S-proteins were then analyzed by standard SDS–polyacrylamide gel electrophoresis and autoradiography with or without previous purification of the mucin domains with TALON-IMAC beads.

Transfection and analysis of recombinant proteins

CHO-K1 and CHO-Lec35.1 cells (kindly provided by Dr. M. A. Lehrman, Texas South Western Medical Center) where maintained in HAMF12 media containing 10% fetal bovine serum (FBS) whereas COS-7 cells were grown in Dulbecco’s modified Eagle medium containing 10% FBS. Cells in 25-cm² flasks were transfected with Eugene-6 (Roche, Indianapolis, IN) as described (Perez-Vilar and Hill, 1997). At 24 h posttransfection, cells were radiolabeled with [35S]-cysteine/methionine as reported previously (Perez-Vilar et al., 1996) except that [35S]-Pro-mix (Amersham Biotech, Little Chalfont, United Kingdom) was used as a source of radiolabeled amino acids. Absorption of His-tagged proteins to TALON-IMAC beads (Clontech) was performed as described (Perez-Vilar and Hill, 1997).
Sigia, St. Louis, MO) (50 mg lectin-agarose, preequilibrated in phosphate buffered saline [PBS] [10 mM sodium phosphate, pH 7.2, 150 mM NaCl] per 2 ml diluted culture medium) for 30 min at 25°C under agitation. The beads were thoroughly washed in PBS; then 50 mM sodium phosphate, pH 7.2, 750 mM NaCl; and, finally, 10 mM Tris–HCl, pH 7.5, prior to boiling the samples in SDS-gel electrophoresis sample buffer. In some experiments, [35S]-proteins bound to lectin-agarose beads were eluted in 0.5 ml 4 M guanidine-HCl in 50 mM Tris–HCl, pH 8.0, and subsequently absorbed to TALON-IMAC beads (20 mg/ml). In other experiments, His-tagged [35S]-proteins bound to TALON-IMAC beads were eluted with PBS containing 10 mM EDTA and absorbed to LCA or GNA-agarose beads (10 mg lectin-agarose per 0.5 ml eluate) as described. Analysis of the proteins by SDS-gel electrophoresis in Tris/glycine or Tris/tricine gels and autoradiography were done using standard methods. 14C-methylated proteins foruplication in the presence or absence of 1 mg/ml bovine serum albumin (Sigma). In some experiments, purified proteins were cross-linked in the presence of 10 mM EDTA, which released proteins from the IMAC resin, or alternatively secreted proteins were cross-linked with BS3 before IMAC purification as described earlier (Perez-Vilar et al., 1996).

Cross-linking studies
Cross-linking of proteins, previously absorbed to TALON-IMAC beads, with BS3 or EDC/S-NHS, both from Pierce (Rockford, IL), were performed essentially as described earlier for BS3 (Perez-Vilar et al., 1996) except that EDC/S-NHS cross-linking was carried out with 1 mM each in 0.1 M 2-(N-morpholino)ethanesulfonic acid, 0.9 % (w/v) NaCl, pH 6.0. For cross-linking with glutaraldehyde, His-tagged proteins bound to TALON IMAC beads were directly reacted with freshly prepared glutaraldehyde (0.01–0.1%, v/v; Sigma) in PBS for 0.5–4 h at 25°C in the presence or absence of 1 mg/ml bovine serum albumin (Sigma). In some experiments, purified proteins were cross-linked in the presence of 10 mM EDTA, which released proteins from the IMAC resin, or alternatively secreted proteins were cross-linked with BS3 before IMAC purification as described earlier (Perez-Vilar et al., 1996).

Live cell imaging
CHO-Lec35.1 cells were subcultured in 35-mm glass-bottom dishes (MatTek, Ashland, MA) and 24 h later transfected with 1 µg DNA and 3 µl Fugene-6 as directed by the manufacturer. After 24 h, the culture medium was changed and the cells incubated for an additional hour. The medium was then replaced with phenol red-free, bicarbonate-free Dulbecco’s modified Eagle medium, 5% FBS, 20 mM HEPES, and observed in a Zeiss LSM 510 (UNC Hooker Microscopy Facility, Chapel Hill, NC) at 37°C in the microscope stage using 488 nm laser excitation for GFP and 413 nm for CFP. Cells clones stably expressing the Cys subdomains were observed after seeding them in the same dishes and once they were 75–85% at confluency. Images were analyzed using Zeiss LSM software.

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Abbreviations
BS3, bis(sulfosuccinimidyl)suberate; CHO, Chinese hamster ovary; CILP, cartilage intermediate layer protein; ECDF, enhanced cyan fluorescent protein; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; EDTA, ethylenediamine tetra-acetic acid; ER, endoplasmic reticulum; FBS, fetal bovine serum; GFP, green fluorescent protein; GNA, Galanthus nivalis agglutinin; LCA, Lens culinaris agglutinin; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; S-NHS, N-hydroxysuccinimide.

References


C-Mannosylation of Cys subdomains