The Epithelial Mucin MUC1 Contains at Least Two Discrete Signals Specifying Membrane Localization in Cells*

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The MUC1 gene product (also designated PEM for polymorphic epithelial mucin, episialin, and DF3) is a high molecular weight, membrane glycoprotein expressed on the apical surface of most simple secretory epithelia. The transmembrane and cytoplasmic domains of MUC1 have been shown to be highly conserved between mammalian species, and it has been shown that this molecule interacts with the actin cytoskeleton. Apical targeting signals in polarized cells have yet to be defined. The mechanism by which MUC1 is targeted and maintained on the apical surface is not known; correct localization, however, would be predicted to be crucial for function. In order to determine which domains of MUC1 were important for this localization, mutational analysis of the protein was undertaken. Using cytoplasmic tail deletion mutants, fusion proteins of MUC1 and CD2, and site-directed mutagenesis, it could be shown that MUC1 appeared to contain at least two motifs involved in apical localization. The first was located in the extracellular domain and was sufficient to confer apical localization on the fusion protein. The second was the Cys-Gln-Cys (CQC) motif at the junction of the cytoplasmic and transmembrane domains. This sequence was necessary for surface expression. These results suggest that MUC1 contains two discrete motifs important in its apical localization.

The MUC1 gene product (also designated PEM for polymorphic epithelial mucin) is a cell-associated glycoprotein expressed on the apical surface of most simple secretory epithelia. The transmembrane and cytoplasmic domains of MUC1 have been shown to be highly conserved between mammalian species, and it has been shown that this molecule interacts with the actin cytoskeleton. Apical targeting signals in polarized cells have yet to be defined. The mechanism by which MUC1 is targeted and maintained on the apical surface is not known; correct localization, however, would be predicted to be crucial for function. In order to determine which domains of MUC1 were important for this localization, mutational analysis of the protein was undertaken. Using cytoplasmic tail deletion mutants, fusion proteins of MUC1 and CD2, and site-directed mutagenesis, it could be shown that MUC1 appeared to contain at least two motifs involved in apical localization. The first was located in the extracellular domain and was sufficient to confer apical localization on the fusion protein. The second was the Cys-Gln-Cys (CQC) motif at the junction of the cytoplasmic and transmembrane domains. This sequence was necessary for surface expression. These results suggest that MUC1 contains two discrete motifs important in its apical localization.

The MUC1 gene encodes a protein that consists of three distinct domains, namely a large extracellular domain and transmembrane and cytoplasmic domains (10–13). The extracellular domain consists predominantly of variable numbers of a 20-amino acid tandem repeat, making the gene encoding MUC1 highly polymorphic, with each allele encoding a product containing different numbers of repeats (10, 14). Each tandem repeat contains several potential sites for O-linked glycosylation, and the mature molecules expressed by the mammary gland have been estimated to be composed of 50–60% carbohydrate (10). The extensive glycosylation of MUC1, combined with the presence of many proline residues, contributes to the extended and rigid structure of MUC1 on the cell surface (15, 16).

It is thought that the cytoskeleton has an important role in maintaining the cytoarchitecture of epithelia and in defining membrane specializations such as junctional complexes and microvilli. Previous work showing that actin-disrupting drugs selectively led to a disruption of the apical localization of MUC1 suggested that interaction with the actin cytoskeleton may be important for its localization (17). Moreover, MUC1 appears to localize to microvilli, and it is possible that it may play a role in organizing the cytoskeleton (17). This is also suggested from the observation that MUC1 is expressed early in developing epithelia before the glands are active, while morphogenesis is still occurring (18). In this paper we attempt to determine if a particular domain of MUC1 contains a signal to specify its apical localization. Discrete domains in the cytoplasmic region of some proteins have been shown to target these proteins to the basolateral domain, although such signals have not yet been identified for apical targeting (19, 20). Because of the high degree of sequence conservation in the cytoplasmic tail of MUC1 in different mammalian species (21–23), we postulated that the cytoplasmic tail is functionally important, and we chose to dissect this domain carefully. One possible function for the cytoplasmic tail is to target MUC1 to the apical membrane. Our results indicated that the cytoplasmic domain was not involved in the apical localization; however, two cysteine residues at the end of the transmembrane/beginning of the cytoplasmic tail appeared to be important for membrane localization. Using chimeric molecules composed of different domains of CD2 and MUC1, we have demonstrated that the extracellular domain of MUC1 can confer apical localization on the CD2 molecule. The results suggest that MUC1 contains two discrete motifs important in its apical localization.

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Tissue Culture Techniques—Cell lines were cultured at 37°C in 10% CO2 in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Cells were passaged every 2–3 days, and early passages were transferred to 3-cm plastic dishes (Nunc) or 16-mm glass coverslips for further processing.

Transfections—MDCK cells were transfected by calcium phosphate precipitation. Cells were grown in 60-mm diameter dishes. Twenty μg of plasmid DNA was precipitated with calcium phosphate and added to the growth medium. Cells were incubated with 4 h at 37°C, followed by incubation with 15% glycerol for 2 min before returning them to normal growth conditions. After 2 h, 0.7 mg/ml G418 was added to the growth medium. Panc-1 cells were transfected by DEAE-dextran precipitation. Cells at 70% confluence were incubated with 20 μg of DNA and 50 μg/ml DEAE-dextran in Tris-buffered saline for 30 min at room temperature. Cells were osmotically shocked with glycerol as above and returned to normal growth conditions. At 72 h post-transfection, cells were grown in selection medium containing 0.6 mg/ml G418. From both cell lines, discrete colonies were isolated and expanded for further testing.

Immunofluorescent Staining—Cells were grown to confluence on 3-cm plastic dishes or glass coverslips. Cells were fixed with 3.5% paraformaldehyde on ice for 15 min and then permeabilized by 0.5% Nonidet P-40 in PBS for 30 min at 4°C. Stained cells were analyzed using a Zeiss Axiophot microscope or a Nikon Optiphot Confocal Microscope.

FACS Analysis—Cells were trypsinized, and 1 × 10^6 cells were processed in microtiter plates for FACS analysis as described (5). Five thousand cells were analyzed on a Becton Dickinson FACS scan.

Electron Microscopy—Cells from a 75-cm^2 flask were scraped gently into a tube and pelleted by centrifugation. For pre-embedding labeling, cells were either unfixed or lightly fixed in 1% monomeric glutaraldehyde in phosphate-buffered saline and fixed further in H2O at 20°C or fixed with 3.5% paraformaldehyde at room temperature. For “live” staining, cells were left unpermeabilized and unfixed and only fixed with methanol-acetone as above at the end of the antibody-labeling procedures. Cells that were solubilized with 0.5% Nonidet P-40 were grown previously in 3-cm plastic dishes for 3–4 days. Cells were incubated on ice for 15 min with 0.5% Nonidet P-40 in 10 mM Hepes, 0.17 M NaCl, 1 mM CaCl2. After a brief washing, cells were fixed with 3.5% paraformaldehyde on ice for 15 min and then further fixed with methanol at −20°C for 15 min. Immunofluorescent staining was as described (23). Stained cells were analyzed using a Zeiss Axioskop microscope or a Nikon Optiphot Confocal Microscope.

APC–Aminopropylamine Microscope—

Oligonucleotides used in construction of MUC1-CD2 chimeras (based on sequences from Refs. 10 and 24). CD2-I, 5'-GAATTCCAAGCTTCTACATGTCCTCG-3' (bp 1074–1094); CD2-B, 5'-GAATTCGCCGGCATCTATCTCATTGGCATATGT-3' (bp 1068–1088); MUC-D, 5'-GAATTCGCCGGCATCTATCTCATTGGCATATGT-3' (638–662); MUC-G, 5'-GAATTCCTCGAGCTGACGGAGCAGCGAG-3' (1297–1329); CD2-F, 5'-GAATTTCCGCGGTGCATCTATCTCATTGGCATATGT-3' (1297–1329).

Development of MUC1 Constructs—A BamHI fragment containing the entire coding sequence of MUC1 including 30 tandem repeats was ligated into the BamHI site of pBS (Stratagene) from which the polylinker sites between Sall and KpnI had been removed by digestion, filled in with Klenow, and religated (creating pBSAscI). This construct had unique AscI and Apal sites in the MUC1 cDNA located 5' and 3' of the transmembrane and CT region (see Fig. 1A for position of restriction sites). MUC1 cDNAs with deletions in the cytoplasmic tail (CT) were created as follows: PCR fragments were generated from the full-length cDNA with a 5' primer spanning the AscI site and 3' primers located at various positions within the CT. The 3' primers contained a translation termination codon and a 3' Apal site. PCR products were subcloned into pBSAscI MUC1 using AscI and Apal. The cDNAs with different length CTs were transferred to phiJaPr-1-neo using BamHI (25).

Site-directed mutagenesis of the cytochrome proximal to the cytoplasmic tail region to alanine was performed using a two-stage PCR method. Overlapping primers containing the necessary nucleotide substitutions were made in both orientations. Using these in combination with a primer at the AscI site (primer A) and a primer at the stop codon (MUC-D), two separate PCR reactions were performed. The PCR products formed the template for the second PCR reaction using the AscI and stop codon primers. The cytochrome were mutated singly (using oligonucleotides AQA M1, AQA M2, or AQA M1, AQC M2) and together (using oligonucleotides AQA M1, AQA M2). After verifying the sequence by sequencing, the PCR product was digested with AscI and BamHI and ligated into similarly cut MUC1 to replace the existing region. All constructs were transferred to phiJaPr-1-neo vector.

Full-length CD2 was subcloned into phiJaPr-1-neo. Constructs containing domains from both CD2 and MUC1 were generated by PCR. Restriction sites were included at the 5' of the PCR primers to allow oligonucleotides from the two molecules without changing the amino acid sequence. All constructs were then ligated into phiJaPr-1-neo expression vector.

To create NOTR and 3TR, an Xmnl to MscI fragment of the MUC1 cDNA spanning the translational start and the entire tandem repeat domain was ligated into the Smal site of pBS in which the AscI site had been destroyed previously (creating pbSTR). Following partial digestion of pbSTR with Smal (which cuts in every tandem repeat), plasmids were recircularized by ligation. Resulting plasmids were analyzed for the number of remaining tandem repeats. DNA fragments containing 3 or 0 tandem repeats were generated by digestion with HindIII and AscI and ligated into phiBSMUC1 replacing the original tandem repeat-containing fragment. Full-length MUC1 cDNAs with 0 or 3 repeats were removed from pBS-AscI by digestion with HindIII and AscI and ligated into phiJaPr-1-neo expression vector. All constructs were verified by extensive restriction enzyme analysis, and fragments generated by PCR were verified by sequence analysis.

RESULTS

Mutant MUC1 Molecules with Deletions of the Cytoplasmic Tail Are Expressed on the Cell Surface—To determine whether the cytoplasmic domain of MUC1 plays an important role in targeting the molecule to the cell surface, truncated forms of the gene were created (Fig. 1A). Stop codons were inserted at specific locations in the cytoplasmic domain coding sequence as outlined under “Experimental Procedures.” The wild type (WT) construct contained the entire extracellular domain including 30 tandem repeats, the transmembrane region, and all 69 amino acids of the cytoplasmic tail. The deletion mutants contained all of the extracellular and transmembrane sequences, and 45 (CT45), 33 (CT33), 18 (CT18), or 3 (CT3) amino acids of...
All constructs were subcloned into the pH transmembrane retention of the molecule during biosynthesis. stop transfer sequence and would therefore be necessary for residues Arg-Arg-Lys (RRK) which are believed to represent a the cytoplasmic tail. The three amino acids were the charged acid of the cytoplasmic tail and indicated.

The membrane localization of MUC1 in the transfected cells was determined by immunofluorescence analysis of stably transfected clones using the antibody HMFG-2 directed to the tandem repeat domain. The cells were grown on glass coverslips for several days in order to form tight monolayers and then stained for immunofluorescence without prior permeabilization and analyzed by confocal laser microscopy. Analysis of the sequence of the XY planes (horizontal sections) of the cells transfected with wild type MUC1 clearly showed segregation of the label to the apical domain of these cells. Vertical XZ sections showed that, in the MDCK cells, which form tight monolayers, the immunofluorescent signal was excluded from the basolateral domain and was seen only in the apical domain (Fig. 2, A and B). In the Panc-1 cells, staining was seen in the apicolateral domain. Panc-1 cells do not form tight monolayers as seen in the MDCK cells and polarize to form apical lateral and basal domains, where the apicolateral domain encompasses much of the lateral region of the cell. MUC1 is restricted to this domain in these cells and is excluded from the basal domain. Similar analysis of both cell lines expressing the deletion mutants CT45, CT33, CT18 (data not shown), and CT3 (Fig. 2, C and D; shown here for Panc-1 CT3 cells) indicated that the mutant MUC1 was expressed on the apical (or apicolateral) cell surface in the same way as the wild type molecule. These results indicated that in these transfected cells the MUC1 protein was targeted to the apical domain and the cytoplasmic tail was not important for apical expression.

Wild Type and Mutant MUC1 Show the Same Ultrastructural Localization—The characteristic punctate pattern of MUC1 observed by indirect immunofluorescence staining and the ultrastructural studies of Parry et al. (17) suggested that MUC1 is associated with the actin cytoskeleton (17), which is well represented in microvilli rich in actin. Electron microscopic immunocytochemistry was carried out on the transfected cells in order to confirm the confocal microscopy results and to determine whether the products of the transfected MUC1 gene and the MUC1 mutants showed the same ultrastructural localization. Ultrastructural studies by Parry et al. (17) have shown that MUC1 in an endogenously expressing cell line was localized to apical microvilli.

Cells were grown to confluence on plastic dishes, scraped from the dishes, and fixed as pellets. Panc-1 cells (which are not as well polarized as MDCK cells) were fixed and embedded prior to staining and immunolabeled with HMFG-2 and gold-conjugated goat anti-mouse IgG, whereas the MDCK cells were fixed and embedded following immunolabeling. This latter type of procedure generally prevents the entry of any labeled anti-
body into intracellular compartments, and only antigen available on the membrane is labeled. Also, since the cells form tight monolayers, they retained their cell-cell attachments during processing, and it was possible to orientate them and to determine the apical and basolateral surfaces, both of which were accessible to labeled antibody. In the MDCK cells, the wild-type MUC1 and CT3 mutant were expressed only on the apical surfaces and appeared to be localized to microvilli as illustrated for CT3 in Fig. 3A. No surface expression was seen in MDCK cells transfected with the vector alone (data not shown). Similar results were observed with the Panc-1 cells. In the cells transfected with empty expression vector, MUC1 was expressed at very low levels both in intracellular vesicles and on the cell surface (Fig. 3B). In the MUC1-transfected Panc-1 cells, a high degree of labeling was observed in intracellular vesicles and on the cell surface (Fig. 3C) where labeling appeared to localize to microvilli. No differences could be detected between the localization of ImmunoGold labeling in transfected Panc-1 cells expressing MUC1 WT and the mutants CT45, CT33, CT18 (data not shown), or CT3 (Fig. 3D) MUC1 proteins. These results demonstrated that the transfected MUC1 was localized to apical microvilli as has been shown for endogenously expressed MUC1 (17), and, similarly, that the CT3 mutant MUC1 was also expressed on microvilli. This suggests that this localization is not dependent on the cytoplasmic tail.

Membrane Retention of MUC1 Is Not Dependent on the Cytosolic Tail—It has been suggested that interactions between MUC1 and the actin cytoskeleton are important for the apical localization of MUC1. Results presented above with the tailless CT3 mutant which is unlikely to be able to interact directly with the actin cytoskeleton suggested that this was not the case. In order to determine whether interactions of the cytoplasmic tail with the cytoskeleton were important for anchoring MUC1 on the cell surface, detergent extracts of cells transfected with MUC1 and the MUC1 mutants were carried out. Extraction of MUC1 from a breast epithelial cell line, MCF-7, with 0.5% Nonidet P-40 for 15 min on ice and subsequent separation by centrifugation of the insoluble and soluble fractions, resulted in approximately 50% of the cellular MUC1 fractionating into the insoluble pellet fraction, as determined by Western blotting. To determine whether interactions of the cytoplasmic tail of the MUC1 gene product with the cytoskeleton were conferring the detergent insolubility on the MUC1 molecule, cells transfected with the wild type and truncated genes were solubilized with 0.5% Nonidet P-40. This concent-

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L. F. Pemberton, A. Rughetti, J. Taylor-Papadimitriou, and S. J. Gendler, unpublished data.
Apical Membrane Localization of Mucin MUC1

The cysteines in this tripeptide motif have been postulated to be involved in the membrane domain adjacent to the cytoplasmic domain of MUC1, which is longer than the most other single spanning domains, and, without fine mapping of this domain, it is not clear whether the CQC residues are actually within the lipid bilayer or within the cytosol. The cysteines in this tripeptide motif have been postulated to be involved in MUC1 protein complex formation (28, 29). In order to determine whether the cysteines in this tripeptide motif were important for MUC1 expression on the apical surface, the cysteine residues were either singly or collectively mutated to alamines using PCR-based site-directed mutagenesis as outlined under "Experimental Procedures." The constructs transfected into MDCK cells were identical with the MUC1 WT construct used in earlier experiments except that one or two cysteine to alanine substitutions (CQA, AQC, or AQA) were encoded (Fig. 5). After transfection and expression in the MDCK cell line, the mutant MUC1 expressing cells were analyzed by indirect immunofluorescence using the antibody HMF-2. Cells were analyzed after methanol:acetone fixation and live, without any prior permeabilization. In the live stained cells, no immunofluorescence was detected in either the CQA, AQC, or AQA mutants, suggesting that MUC1 was not present on the cell surface (Fig. 6, Column 1). In the fixed cells, however, strong immunofluorescence could be detected in a diffuse pattern in the cytoplasm, with some signal localizing to the lateral regions of the cell (Fig. 6, Column 2). These results demonstrated that single amino acid substitution of either cysteine of the CQC tripeptide results in a loss of surface expression and suggested that this domain may be important for apical targeting or retaining MUC1 on the cell surface.

Analysis of the Domains of MUC1 Important for Apical Expression—As no portion of the cytoplasmic tail had been demonstrated to be responsible for the apical targeting and retention of MUC1, it was possible that, in addition to the CQC motif, the transmembrane or extracellular domains contained signals that were important for apical localization. To analyze this further, chimeric proteins were constructed as described under "Experimental Procedures." Fusion proteins consisting of the lymphocyte-specific glycoprotein CD2 and specific regions of the MUC1 protein were constructed to determine which domain of MUC1 conferred apical localization on CD2. CD2 was chosen as a reporter molecule as it is expressed only on nonpolarized lymphoid cells (30) and would not be expected to contain strong targeting signals.

Three chimeric proteins were made (Fig. 7). The construct called TMCT consisted of the CD2 extracellular domain and the MUC1 transmembrane and cytoplasmic domains. A second construct, TM, consisted of the CD2 extracellular domain and the transmembrane domain of MUC1 with the three charged stop transfer amino acids, RRK. A third construct, designated MEX, consisted of the MUC1 extracellular domain containing...
30 tandem repeats fused to the transmembrane and cytoplasmic tail domains of CD2. The entire coding region of CD2 was also cloned into the expression vector. The three chimeric proteins and the wild type CD2 and MUC1 proteins were expressed in stable transfectants of MDCK cells. After transfection and selection of individual clones, the cells were processed for immunofluorescence staining and analyzed by laser scanning confocal microscopy. The MDCK cells expressing wild-type CD2, TM, and TMCT were stained with the antibody OKT11 reactive with an epitope in the extracellular domain of CD2. Cells were fixed with methanol:acetone prior to labeling, since the staining was much stronger following fixation, which presumably allowed access of the antibody to its epitope. The distribution of the protein was visualized in the XY (horizontal, Fig. 8, Column 1) and the XZ (vertical, Fig. 8, Column 2) planes. In the horizontal plane, the labeling pattern appeared as a sharp profile outlining cell-cell contact areas, in a characteristic basolateral pattern. Small amounts of stained intracellular material were also visible. In the XZ plane, the label appeared to be restricted to the lateral surfaces of the cells (Fig. 8, A, B, and E–H). Therefore, in these cells, the wild type CD2 protein was expressed exclusively on the basolateral domain of the cells. Comparison of at least three clones for each of the CD2, TM, and TMCT transfectants showed that no significant differences in the staining patterns could be detected; all the proteins were expressed basolaterally. Similar results were obtained when the cells were analyzed without prior fixation, although the staining was weaker.

The MDCK cells expressing the wild type MUC1 and MEX proteins were not fixed prior to labeling and were labeled with the monoclonal antibody HMFG-2 for confocal analysis. The staining pattern for wild type MUC1 transfectants was as described in Fig. 3 with the characteristic apical staining seen in the XY section (Fig. 8, C and D). In the cells expressing the MEX chimeric protein, the staining pattern was the same, with punctate dots appearing over the apical surface of the cell (Fig. 8, I and J). In the vertical sections, both the wild type MUC1 and the MEX chimera exhibited labeling that was restricted to the apical domain (Fig. 8, D and J).

These results suggested that the extracellular domain was the region important for the apical targeting of the fusion proteins. Therefore, we asked whether the tandem repeat domain was determining this localization, since this domain comprises much of the extracellular portion of the MUC1 protein. Two constructs were made and expressed in MDCK cells (Fig. 9). The first, called NOTR, consisted of the MUC1 protein containing no tandem repeats but with the flanking degenerate 5’ and 3’ repeat units intact. The second construct, designated 3TR, consisted of the entire MUC1 sequence with three tandem repeats of 20 amino acids in the extracellular region. Following transfection of MDCK cells and selection of individual clones, immunofluorescence analysis was performed as before. Since

![Figure 6](http://www.jbc.org/)

**Fig. 6.** Cellular localization of the MDCK CQA, AQC, and AQA mutants. MDCK cells transfected with the AQC (A and B), CQA (C and D), or AQA (E and F) constructs were stained by indirect immunofluorescence using the antibody HMFG-2. Cells were either not permeabilized prior to staining (column 1) or fixed in methanol:acetone (column 2). Magnification × 630.

![Figure 7](http://www.jbc.org/)

**Fig. 7.** MUC1 and CD2 chimeric constructs. A schematic representation of chimeric constructs made by PCR using sequences from MUC1 (gray shading, hatched area represents tandem repeat domain) and CD2 (black shading). The extracellular, transmembrane, and cytoplasmic domains are indicated, and names shown are also those used for cell lines transfected with these constructs. 3TR and NOTR represent MUC1 constructs that encode for MUC1 with only 3 (3TR) or no (NOTR) tandem repeats. Both constructs include the remainder of the MUC1 coding sequence.
Apical Membrane Localization of Mucin MUC1

**DISCUSSION**

MUC1 gene product is detected on the apical surface of most simple glandular epithelia. The precise function of this protein is not well understood, although it is probable that apical localization is important for its function. In this paper we have analyzed the contribution of the various domains to the apical targeting of the molecule by transfecting into polarized epithelial cells mutated, deleted, or chimeric forms of the MUC1 gene. Deletions of the cytoplasmic tail of MUC1 appeared to have no effect on the apical localization and, surprisingly, MUC1 with virtually no cytoplasmic tail was shown not only to be correctly localized to the apical microvilli, but also retained in the detergent insoluble fraction. In contrast to the lack of effect seen by removing the entire cytoplasmic domain, the substitution of a single transmembrane/cytoplasmic cysteine by an alanine led to the protein being mislocalized, with no MUC1 detected on the cell surface. However, while these cysteines appear to be important for membrane localization of the full-length MUC1, their presence in the fusion proteins discussed below is not sufficient to confer apical localization in cells transfected with these hybrid genes.

Hybrid molecules containing the extracellular domain of CD2 and the transmembrane or transmembrane and cytoplasmic domains of MUC1, although expressed on the cell surface, were not localized to the apical domain but to the basolateral domain. These results indicated that both the cytoplasmic and transmembrane domains lacked dominant signals for apical targeting. It was possible that CD2 contained a basolateral targeting signal in the extracellular domain; however, this seems unlikely as it is endogenously expressed on nonpolarized cells, and all basolateral signals identified to date have been cytoplasmically located (19, 20). In contrast, a fusion protein consisting of the extracellular domain of MUC1 and the transmembrane and cytoplasmic domains of CD2 was targeted to the apical surface, suggesting that the extracellular domain of MUC1 was the domain required for apical targeting.

The transmembrane and cytoplasmic domains of MUC1 are conserved between mammalian species with an amino acid identity of 80–90% (22, 23), suggesting that this domain is functionally important in the MUC1 molecule as a whole. The cytoplasmic tail, which has been described as being phosphorylated, contains 7 tyrosines, 6 of which are conserved in other species (22, 29). MUC1 has been shown to be recycled through the trans-Golgi network from the apical surface several times (32), a process that is accompanied by further sialylation of the O-glycans. A specific endocytic determinant has not been shown in MUC1, but, by analogy with other proteins, one of the cytoplasmic tyrosines could be involved (33). Although the cytoplasmic tail may be involved in endocytosis, our results suggest that this function is not necessary for maintaining the apical localization of MUC1.

The association of MUC1 with the actin cytoskeleton is implied from the capping effect of cytochalasin D (17), and the cytoplasmic domain would be the most likely candidate. We
postulated that this interaction may be important for the correct localization of MUC1. However, as our results showed clearly that the cytoplasmic domain was not necessary for apical localization nor for the detergent insolubility and retention of MUC1 in the membrane, it is unlikely that MUC1–cytoskeletal interactions are important for this localization. The detergent insolubility was most likely not due to direct cytoskeletal interaction. Thus, it seems likely that some other domain of the MUC1 molecule is important for anchoring it on the apical surface. Similar results using a mutant version of CD44 with a deleted cytoplasmic domain demonstrated that a significant fraction of this mutant also partitions into the detergent insoluble fraction (34). Interestingly, a cytoplasmic deletion mutant of MUC1 has been shown to be able to be capped using cross-linked antibodies (8). It is possible that MUC1 is forming large detergent insoluble complexes either with itself or another molecule, perhaps via transmembrane or extracellular interactions.

The cysteines in the tripeptide motif CQC have been postulated to be involved in the formation of protein complexes (28). Studies involving the small molecule MUC1Y showed these complexes were detected only under nonreducing conditions (29, 35). This suggests that disulfide bond formations could be involved, although this is unlikely as these cysteines are cystolic and not exposed to the lumen of the ER. A similar CXC motif in the cytoplasmic domain of CD4 has been shown to be involved in complex formation (36). The authors suggested these cysteines were involved in forming protein complexes involving heavy metals such as zinc. Further studies are needed to distinguish more precisely the mechanism by which the CQC motif is functioning.

Although all basolateral targeting signals investigated to date have been shown to comprise short motifs of several amino acids, usually including tyrosines located within the cytoplasmic domain, similar sorting determinants for apical proteins have not been identified (19, 20). The identification of the Drosophila protein crumbs as an apical protein that promotes the development of the apical membrane provides direct evidence of an apical targeting signal (37). One group of specialized proteins that are anchored in the membrane by a glycosylphosphatidylinositol anchor are expressed apically, and the glycosylphosphatidylinositol anchor has been shown to confer apical localization on these proteins (38). For some viral proteins detected on the apical membrane of cells after infection, the sorting determinant has been shown to be in the ectodomain (39). Other proteins that are usually localized to the apical membrane are selectively secreted from the apical surface when expressed as truncated proteins lacking a transmembrane or cytoplasmic domain, also suggesting that there may be a sorting determinant in the ectodomain (40, 41). Many investigators have suggested that the transport machinery for apical and basolateral proteins differs. Antibodies against NSF and N-nitroso-DL-penicillamine (SNAP) affect only the basolateral pathway (42). Some studies have suggested a differential requirement for heterotrimeric G proteins and small GTP-binding proteins in the sorting and fusion of apically and basolaterally bound vesicles (43, 44). Prevention of GDP exchange on Rab proteins using a Rab-GDI affects only the basolateral pathway (42), while others have shown that the kinases, cAMP-dependent protein kinase and protein kinase C, selectively stimulate the apical transport pathway (45). It has also been demonstrated that the transport of basolaterally and apically destined vesicles has a differential requirement for microtubule-based motors (46). Apical and basolateral transport is mediated by distinct sets of transport vesicles (47), and apical transport vesicles themselves have been shown to be enriched in different subsets of proteins such as annexin XIIIb (48). It is likely that proteins carried in these vesicles destined for the apical domain possess some determinant to mark them as apical so that enrichment in the correct vesicle and engagement of the correct machinery can occur. As discussed above, it is possible that this signal is contained in the ectodomain of the protein. If so, it may depend on conformation, since homology has not been observed in the ectodomains of apically targeted proteins. Therefore, the inclusion of a determinant in the extracellular domain such as we have described for MUC1 is consistent with this idea.

As the construct lacking the tandem repeat domain (NOTR) was expressed on the apical membrane, it appears that the signal for apical localization and retention in the apical membrane is not contained within the tandem repeats. The tandem repeat domain is a scaffold for the majority of the O-linked carbohydrate that is attached to MUC1 (10). It is, therefore, probable that this O-linked carbohydrate is not playing a role in the localization either. Unpublished data from our laboratory indicate that inhibition of addition of the O-linked carbohydrate does not affect the localization of MUC1. However, it has been suggested by Fiedler and Simons (49) that N-linked glycans may affect the apical localization of some proteins, and there are a number of sites for both N- and O-linked glycosylation outside the tandem repeat domain. N-Linked glycosylation of MUC1 has been demonstrated (50). Studies are in progress to determine the significance of N-linked carbohydrate in apical trafficking of MUC1.

Comparison of the extracellular domain from different species shows a much lower level of homology than is observed in the transmembrane and extracellular domains (21, 22). Analysis of partial MUC1 clones from several mammalian species that include the extracellular domains proximal to the membrane indicated that, although homology overall was low, several short motifs were well conserved, and it is possible these may represent sequences important in the apical localization of MUC1 (22).

In summary, although the cytoplasmic domain is highly conserved, it is not important in the localization and retention of MUC1 at the apical cell surface. In contrast, the molecule appears to contain at least two motifs involved in this localization. One is at the junction of the transmembrane and cytoplasmic domains that is necessary for surface expression, and the other is in the extracellular domain (outside of the tandem repeat region). A more detailed analysis of the stalk region of the extracellular domain which contains sequences conserved across species is in progress.

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