Tumor Necrosis Factor-α Converting Enzyme/ADAM 17 Mediates MUC1 Shedding*

Amantha Thathiah‡, Carl P. Blobel§, and Daniel D. Carson‡¶

From the ‡Department of Biological Sciences, University of Delaware, Newark, Delaware 19716 and the §Cellular Biochemistry and Biophysics Program, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

MUC1 clearance from the uterine epithelial cell surface is a prerequisite for the creation of an environment conducive to embryo implantation. In some species, reduced mRNA levels along with metabolic turnover account for loss of MUC1 during the receptive phase throughout the uterine epithelium. In other species, MUC1 is rapidly lost solely at the site of blastocyst attachment, suggesting the action of a protease. Correlative studies also indicate the presence of soluble forms of MUC1 in cell culture supernatants in vitro and in bodily fluids in vivo. To characterize the proteolytic activity mediating MUC1 release, shedding of MUC1 was analyzed in a human uterine epithelial cell line (HES) that abundantly expresses and readily sheds MUC1. MUC1 release was stimulated by phorbol 12-myristate 13-acetate and was markedly inhibited by the synthetic peptide hydroxamate metalloprotease inhibitor, tumor necrosis factor-α protease inhibitor (TAPI), as well as by an endogenous inhibitor of matrix metalloproteases, tissue inhibitor of metalloproteases (TIMP)-3. These characteristics along with studies conducted with cell lines genetically deficient in various ADAMs (for a disintegrin and metalloprotease) identified tumor necrosis factor-α converting enzyme (TACE)/ADAM 17 as a MUC1 sheddase. Furthermore, both TACE and MUC1 were expressed in human uterine epithelia during the receptive phase, and co-immunoprecipitation experiments revealed a physical interaction between TACE and MUC1 in HES cells. These studies establish a proteolytic mechanism for MUC1 clearance from a human uterine epithelial cell line and identify TACE as a MUC1 sheddase.

Proteolytic removal of the extracellular domain of numerous transmembrane proteins is responsible for the regulated release of cytokines, growth factors and their receptors, cell adhesion molecules, and ectoenzymes (reviewed in Refs. 1–3). This process, referred to as ectodomain shedding, appears to be essential for mammalian development (4) and has implicated roles in leukocyte migration, cell-cell adhesion, and tumor cell proliferation. Ectodomain shedding also is associated with the progression of several disease processes, including rheumatoid arthritis (5), other autoimmune inflammatory syndromes (6), and Alzheimer’s disease (Ref. 7; reviewed in Ref. 8). Consequently, identification of the proteases or “sheddases” involved in the release of transmembrane proteins should provide insight into the regulation of key physiological events under normal and pathological conditions. In this regard, members of the ADAM1 (for a disintegrin and metalloprotease) family of metalloproteases appear to mediate the ectodomain release of several proteins (reviewed in Refs. 3, 9, and 10). Tumor necrosis factor (TNF)-α converting enzyme (TACE)/ADAM 17, initially identified as the catalytic activity responsible for proteolytic processing of TNF-α (11, 12), also is involved in or has been implicated in the ectodomain release of transforming growth factor-α, L-selectin, p75 TNF receptor (p75 TNFR) (4), β-amyloid precursor protein (βAPP) (13), p55 TNFR, interleukin-1 receptor (IL-1R) II (14), erbB4/HER4 (15), the Notch1 receptor (16), IL-6R (17), growth hormone-binding protein (18), cellular prion protein (19), and fractalkine (20, 21). Moreover, mice that are genetically deficient in catalytically active TACE display an embryonic lethal phenotype (4), which underscores the importance of TACE-mediated shedding in vivo and suggests an essential role for ADAMs in protein ectodomain release. In light of these findings and others, numerous in vitro studies attribute proteolytic processing of membrane-anchored proteins to one or more ADAMs.

Embryo implantation is a highly regulated process that requires both an attachment competent blastocyst and a receptive uterus. The initial stage of implantation is mediated by interactions between the apical surface of the uterine epithelium and the trophoderm of the blastocyst. Under most conditions, however, the apical surface of the uterine epithelium is protected by a thick glyocalyx composed largely of mucins. MUC1, a transmembrane mucin and an important component of the glyocalyx, provides a physical barrier to microbial and enzymatic attack (22, 23). MUC1 exerts its anti-adhesive effect through a large extracellular domain primarily composed of a series of 20-amino acid repeats enriched in serine, threonine, and proline residues (24). The numerous proline residues and extensive O-linked glycosylation on serine and threonine residues generate a highly extended and rigid structure that protrudes 200–500 nm into the pericellular space (25). As a result, a major challenge that the uterus faces...
during the receptive phase is to maintain this protective barrier while permitting blastocyst attachment. During the receptive phase, and in response to ovarian steroid hormones, MUC1 expression is reduced throughout the uterine epithelium in several species (26–29), but is elevated in rabbits and humans (30, 31). In both instances, global changes in MUC1 expression correlate with changes in MUC1 mRNA levels. However, the presence of the blastocyst in the rabbit endometrium results in a localized reduction of MUC1 at the site of implantation (30, 31). Coincidently, ADAM 9 accumulates at sites of blastocyst attachment (and MUC1 loss) (32), implicating a role for this ADAM in the implantation process in rabbits. An in vitro study of cultured human uterine epithelial cells similarly demonstrated a local loss of MUC1 at the site of human blastocyst attachment (33). This finding is consistent with an induced loss of MUC1 at the site of attachment, perhaps mediated through activation of a cell surface protease. The aim of the current study was to initially characterize the mechanism of MUC1 cell surface release. We provide evidence that MUC1 is cleaved from the surface of a human uterine epithelial cell line, HES, by a protease(s) that is stimulated by phorbol 12-myristate 13-acetate (PMA) and is inhibited by the hydroxamate-based metalloprotease inhibitor, TAPI, and the tissue inhibitor of metalloproteases (TIMP)-3. We identify TACE as a mediator of constitutive and phorbol ester-stimulated MUC1 shedding. In addition, we demonstrate that both TACE and MUC1 are expressed in human uterine epithelia during the receptive phase in vivo and form a stable physical association in HES cells in vitro.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phorbol-12-myristate 13-acetate and the furin inhibitor, decanoyl-RVKR-CMK, were purchased from Calbiochem. Leupeptin, pepstatin A, and E-64 were obtained from Sigma. TIMP-1, TIMP-2, TIMP-3, rabbit anti-TACE antibody, and anti-TACE blocking peptide were obtained from Chemicon. Protein G-Sepharose, Texas Red-conjugated sheep anti-mouse IgG, and fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG were purchased from Amersham Biosciences. 4,6-Diamidino-2-phenylindole, dihydrochloride (DAPI), was purchased from Molecular Probes. Affinity-purified mouse IgG and rabbit IgG were obtained from Zymed Laboratories Inc. Mouse monoclonal antibody specific for a tandem repeat epitope in the extracellular domain of MUC1, 214D4, was kindly provided by Dr. John Hilkens (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The metalloprotease inhibitor, TAPI, was kindly provided by Dr. John Doedens and Dr. Roy Black (Immunoex, Seattle, WA).

**cDNA Constructs**—The TACE-FLAG cDNA was kindly provided by Dr. John Doedens. The full-length human MUC1 cDNA, the generous gift of Dr. Sandra Gendler (Mayo Clinic, Scottsdale, AZ), was cloned into the expression vector pcDNA3.1 (Invitrogen) to allow for expression of MUC1.

**Reverse Transcription-PCR**—Total RNA was extracted from HES cells using the RNeasy kit according to instructions from the manufacturer (Qiagen). Total RNA was extracted from frozen human endometrial tissue samples using the TRIzol reagent according to instructions from the manufacturer (Invitrogen). Quantitation and estimation of purity were performed by measuring the absorbance of each RNA sample at UV wavelengths of 260 and 280 nm, and integrity was determined by visual inspection of RNA fractionated by agarose gel electrophoresis. Reverse transcription was performed using the Advantagewhite RT-for-PCR kit according to instructions from the manufacturer (Clontech). PCR was performed using the HotStarTaq Master Mix kit according to instructions from the manufacturer. The Advantage RT-for-PCR kit was used to amplify cDNA from human endometrial tissue samples using the following primers: MUC1 sense (5’-GAGGCTGGATTCCTGCACT-3’) and MUC1 antisense (5’-GATAGTTCTCTTGCTGAGCC-3’). PCR reactions were performed in 1% agarose gel electrophoresis. A 500-bp product was detected by the manufacturer. Signal intensities were measured with the one-dimensional Multi Alpha Imager program (Alpha Innotech). Statistical analyses were performed using one-way analysis of variance and the Tukey-Kramer multiple comparisons test (GraphPad InStat program).

** SDS-PAGE and Detection of MUC1 Protein**—Cell-associated and culture supernatant protein samples were separated by SDS-PAGE using 10% acrylamide gel stacking gel and a 10% acrylamide gel resolving gel (34, 40). The samples were transferred to a nitrocellulose membrane (Schleicher & Schuell, 0.45 μm) and blocked with 5% nonfat milk in tris-buffered saline (TBS) (20 mm Tris, pH 7.5, 150 mm NaCl, 0.1% Tween 20) for 1 h at 4 °C and probed with 1:500 dilution of mouse monoclonal antibody 214D4 (39), or nonimmune control immunoglobulins. The antigen-antibody complexes were visualized using the one-dimensional Multi Alpha Imager program (Alpha Innotech). Signal intensities were measured with the one-dimensional Multi Alpha Imager program (Alpha Innotech). Statistical analyses were performed using one-way analysis of variance and the Tukey-Kramer multiple comparisons test (GraphPad InStat program).
Ectodomain Shedding of MUC1 by TACE

RESULTS

MUC1 Release Is Rapidly Stimulated by PMA—Shedding of various cell surface proteins has been shown to be stimulated by phorbol esters, such as PMA (20, 41–43), presumably through activation of metalloproteolytic sheddases. Therefore, we tested PMA for induction of MUC1 release from HES cells. HES cells constitutively release MUC1, and as shown in Fig. 1, A (upper panel) and B, following PMA stimulation, release was rapidly enhanced at least 6-fold; however, PMA stimulation did not detectably alter cell-associated MUC1 levels (Fig. 1 A, lower panel).

To confirm that the soluble MUC1 observed in the supernatants of HES cells contained ectodomains only, cell lysates and conditioned medium from PMA-stimulated and unstimulated cells were immunoprecipitated with a polyclonal antibody, CT-1, specific for the cytoplasmic domain of MUC1 or with a monoclonal antibody, 214D4, specific for the extracellular domain of MUC1 and then examined by Western blot analysis, probing the membrane with the monoclonal, ectodomain antibody (Fig. 2). Concurrent with similar findings in the HES and other cell lines (37, 44, 45), soluble MUC1 from HES cells was immunoprecipitated with 214D4, but not with CT-1, indicating that MUC1 in the supernatants of unstimulated and PMA-stimulated HES cells lacks the cytoplasmic tail. Previous work
has demonstrated that MUC1 constitutively released from HES is not associated with particulate elements, i.e. membrane “blebs,” and that constitutive cell surface release of biotinylated MUC1, devoid of the cytoplasmic tail, accompanies the gradual disappearance of cell-associated, biotinylated MUC1, immunoprecipitable with both ectodomain and cytoplasmic domain MUC1 antibodies (37). Thus, the presence of ectodomain fragments in the culture supernatants from untreated cells indicates that MUC1 is released in the absence of a stimulus, and enhanced detection of MUC1 following PMA treatment is the result of increased release, rather than membrane blebbing.

A Metalloprotease Is Responsible for Shedding of MUC1—To initially characterize the activity mediating MUC1 cell surface release, a series of protease inhibitors were examined for their ability to modulate MUC1 shedding. Inhibitors of serine (leupeptin), cysteine (E-64), and aspartyl (pepstatin A) proteases had no effect on constitutive or PMA-stimulated release of MUC1 (Table I). In contrast, the hydroxamate-based metalloprotease inhibitor, TAPI, completely inhibited PMA-stimulated release (Fig. 3A) and also substantially diminished constitutive MUC1 release, an effect that was more readily observable at later time points (data not shown).

To further characterize the MUC1 sheddase(s), we examined the ability of endogenous metalloprotease inhibitors, TIMP-1, -2, and -3 (reviewed in Refs. 46 and 47), to inhibit MUC1 shedding. At concentrations known to inhibit MMPs (48) and ectodomain release of Her2 (49) and TRANCE (34), TIMP-1 and TIMP-2 failed to inhibit MUC1 release from HES cells (Table I). Interestingly, TIMP-2 actually stimulated MUC1 shedding. However, TIMP-3, unique in its ability to inhibit TACE/ADAM 17 (50), significantly inhibited PMA-enhanced shedding of MUC1 (Fig. 3B).

Prodomain removal during biosynthesis is a prerequisite for protease activity of catalytically active ADAMs and appears to be mediated by a furin-type proprotein convertase in the trans-Golgi network (42, 51). Consequently, inhibition of furin might potentially prevent prodomain removal of the MUC1 sheddase(s) and subsequently inhibit activation. In this regard, decanoyl-Arg-Val-Lys-Arg (decanoyl-RVKR), a furin inhibitor (52), was tested for its ability to modulate MUC1 ectodomain release. Interestingly, decanoyl-RVKR was effective at inhibiting PMA-induced MUC1 release (Fig. 3C), suggesting that the MUC1 sheddase(s) is processed by a metalloprotease sensitive to furin or a furin-like proprotein convertase. Collectively, these data were consistent with a role for an ADAM(s) mediating MUC1 shedding.

HES Cells Express a Majority of ADAMs with a Catalytic Consensus Sequence—To address the potential role of an ADAM(s) in MUC1-mediated shedding, we determined the expression of ADAMs that are widely expressed and contain a catalytic consensus sequence in HES cells and in the receptive phase human endometrium by reverse transcription-PCR. ADAMs 8, 9, 12, 15, 17, 19, and 33 were expressed at the mRNA level in the HES cells and in the receptive phase human endometrium (Table II). However, ADAM 28 only appeared to be expressed in the receptive phase human endometrium. We did not examine expression of ADAM 10 in the HES cells or in the endometrium because the MUC1 sheddase(s) was not inhibited by TIMP-1, and TIMP-1 has been shown to inhibit ADAM 10 activity (53).

MUC1 Is Not Shed by tace<sup>ΔZn/ΔZn</sup> EC-2 Cells—Isolation of cells from ADAM-deficient mice has permitted direct evaluation of proteases and their potential substrates (4, 13–16, 20, 21, 34, 54). Wild-type EC-4 and tace<sup>ΔZn/ΔZn</sup> EC-2 cells are immortalized embryonic fibroblasts derived from wild-type and tace<sup>ΔZn/ΔZn</sup> mice (4). We utilized these cell lines to assess the importance of TACE in MUC1 ectodomain release. Neither the wild-type EC-4 nor the tace<sup>ΔZn/ΔZn</sup> EC-2 cells express MUC1 endogenously. Therefore, MUC1 shedding was examined fol-

### Table I
Protease inhibitor insensitivity of constitutive and PMA-stimulated MUC1 shedding

| Inhibitor | Protease class | Concentration | Inhibition Constitutive | Inhibition PMA-stimulated |
|-----------|----------------|--------------|-------------------------|---------------------------|
| Leupeptin | Serine         | 10 μM        | 0                       | 0                         |
| Pepstatin A | Aspartyl     | 10 μM        | 0                       | 0                         |
| E-64     | Cysteine      | 10 μM        | 0                       | 0                         |
| TIMP-1   | Metalloprotease | 20 μg/ml     | 0                       | 0                         |
| TIMP-2   | Metalloprotease | 20 μg/ml     | 0                       | 0                         |

### Fig. 3

PMA-induced MUC1 release is sensitive to the metalloprotease inhibitor, TAPI, the endogenous metalloprotease inhibitor, TIMP-3, and the furin inhibitor, decanoyl-RVKR. Following pretreatment with TAPI, TIMP-3, or decanoyl-RVKR for 1 h at 37°C, HES cells were treated with PMA or vehicle in the presence or the absence of TAPI (A), TIMP-3 (B), or decanoyl-RVKR (C) for 1 h at 37°C. MUC1 recovered from culture supernatants was then examined by Western blot analysis. Experiments were performed in triplicate and repeated three times with similar results.

A. PMA: + + +, TAPI: + + +, 205kDa

B. PMA: + + +, TIMP-3: + + +, 205kDa

C. PMA: + + +, F.I.: + + +, 205kDa

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lowing electroporation of these cells with MUC1 cDNA or empty vector. Transfected EC-4 cells behaved similarly to HES cells with respect to constitutive and PMA-induced release of MUC1 (Fig. 4A). Furthermore, PMA-stimulated MUC1 shedding was sensitive to inhibition by TAPI. In contrast, constitutive shedding in the TACE populations, demonstrating that TACE is the predominant enzyme responsible for the levels of cell-associated MUC1 were identical in the two cell lines (44) and normal mouse uterine epithelial cells (38) demonstrated expression of both TACE and MUC1 in these cell populations, indicating that TACE is the predominant MUC1 sheddase in this system for constitutive as well as stimulated MUC1 release. To confirm that the lack of MUC1 shedding in the tace−/− fibroblasts was the result of the absence of TACE, we co-transfected the TACE-deficient fibroblasts with MUC1 and TACE cDNA. As shown in Fig. 4C, both constitutive and PMA-stimulated MUC1 shedding were restored in the co-transfectants. Taken together, these results demonstrate the critical importance of TACE in MUC1 shedding in this system.

ADAMs 9, 12, and 15 Are Not Required for MUC1 Shedding—To analyze the importance of other ADAMs in MUC1 shedding, we assessed MUC1 ectodomain release in primary embryonic fibroblasts from ADAM 9/12/15-deficient mice. The wild-type and ADAM 9/12/15-deficient fibroblasts express comparable levels of MUC1 following transfection with MUC1 cDNA (Fig. 5). Moreover, both cell lines shed MUC1 to a similar or identical extent, arguing against a role for these ADAMs in MUC1 ectodomain release.

Expression of MUC1 and TACE in HES Cells—It was considered that a stable physical interaction might occur between TACE and MUC1 prior to MUC1 cleavage. MUC1 was immunoprecipitated from HES cells with either 214D4 or CT-1, which recognize epitopes in the ectodomain and cytoplasmic domain of MUC1, respectively. Immunoprecipitates were then examined by Western blot analysis for TACE (Fig. 6). A Jurkat cell lysate served as a positive control for recognition of TACE. The bands detected most likely represent precursor, glycosylated, and mature forms of TACE. Preincubation of the anti-TACE antibody with the peptide to which it was generated effectively eliminated the appearance of these bands (data not shown). Thus, regardless of the antibody used to immunoprecipitate MUC1, TACE was detected in all samples except for the IgG negative control immunoprecipitates, indicating a physical association between MUC1 and TACE occurs in HES cells. Interestingly, PMA stimulation appears to increase the association between TACE and MUC1 in cells that have been immunoprecipitated with CT-1 (Fig. 6, lanes 5 and 6), whereas this interaction is diminished following PMA stimulation in cells that have been immunoprecipitated with 214D4 (Fig. 6, lanes 7 and 8).

Localization of MUC1 and TACE in the Receptive Phase Human Endometrium—We next considered whether TACE was expressed appropriately in the human uterus to participate in MUC1 shedding. Therefore, TACE and MUC1 expression were examined in tissue sections from the receptive phase human endometrium by fluorescence microscopy. Consistent with previous findings in other systems (42, 55, 56), TACE displayed an intracellular and perinuclear distribution in luminal and glandular uterine epithelial cells with barely detectable staining in the surrounding stromal cells (Fig. 7). Notably, TACE is expressed at the apical aspect of luminal and glandular uterine epithelial cells, which is the predominant site of MUC1 localization (Fig. 7, A, D, and J). The staining pattern for MUC1 was not observed with a nonimmune control antibody (Fig. 7K), and preincubation of the anti-TACE antibody with the peptide to which it was generated effectively blocked cellular staining (Fig. 7G). Consequently, this demonstrated expression of both TACE and MUC1 in epithelia of the receptive phase human endometrium.

**DISCUSSION**

MUC1 plays a critical role in embryo attachment, bacterial clearance, and various aspects of tumor progression. A high level of MUC1 expression effectively inhibits cell-cell (57) and cell-extracellular matrix (39) adhesion. In addition, a correlation has been observed between overexpression of MUC1, the metastatic potential of primary tumors, and poor patient survival (58–60). Interestingly, several findings indicate the presence of soluble MUC1 fragments in bodily fluids, implying that proteolytic shedding takes place in vivo (45, 61). Consistent with these findings, studies conducted with breast cancer cell lines (44) and normal mouse uterine epithelial cells (38) demonstrated that, during normal metabolic turnover, a significant portion of released cell surface MUC1 lacks the cytoplasmic tail. Findings in breast, colon, and pancreatic cancer cells, in addition to normal mammary and prostate, and in HES uterine epithelial cells also indicate that an intracellular/metabolic proteolytic cleavage occurs that separates the extracellular domain from the transmembrane and cytoplasmic tail domains during assembly (37, 62, 63). The MUC1 heterodimer, consisting of the two proteolytic cleavage products, remains tightly,
MUC1 shedding does not occur in tace<sup>−/−</sup> EC-2 cells. A, embryonic fibroblasts derived from wild-type (EC-4) mice were transiently transfected either with cDNA encoding full-length MUC1 (lanes 3–6) or with the vector control (lanes 1 and 2) and treated with PMA or vehicle in the presence or absence of TAPI for 2 h. MUC1 in culture supernatants (upper panel) and cell lysates (lower panel) then was examined by Western blot analysis. Experiment was performed in duplicate and repeated three times with similar results. B, MUC1 shedding from EC-2 embryonic fibroblasts derived from tace<sup>−/−</sup> mice was determined as described for wild-type EC-4 cells in A. C, EC-2 cells were co-transfected with cDNA encoding full-length MUC1 and TACE (lanes 3 and 4) or vector controls (lanes 1 and 2). Cells were treated with PMA or vehicle for 2 h. MUC1 recovered from the culture supernatants (upper panel) and in the cell lysates (lower panel) was assessed as described for wild-type EC-4 cells in A.

![Figure 4](Image)

**Fig. 4.** MUC1 shedding does not occur in tace<sup>−/−</sup> EC-2 cells. A, embryonic fibroblasts derived from wild-type (EC-4) mice were transiently transfected either with cDNA encoding full-length MUC1 (lanes 3–6) or with the vector control (lanes 1 and 2) and treated with PMA or vehicle in the presence or absence of TAPI for 2 h. MUC1 in culture supernatants (upper panel) and cell lysates (lower panel) then was examined by Western blot analysis. Experiment was performed in duplicate and repeated three times with similar results. B, MUC1 shedding from EC-2 embryonic fibroblasts derived from tace<sup>−/−</sup> mice was determined as described for wild-type EC-4 cells in A. C, EC-2 cells were co-transfected with cDNA encoding full-length MUC1 and TACE (lanes 3 and 4) or vector controls (lanes 1 and 2). Cells were treated with PMA or vehicle for 2 h. MUC1 recovered from the culture supernatants (upper panel) and in the cell lysates (lower panel) was assessed as described for wild-type EC-4 cells in A.

but noncovalently, associated at the cell surface through a SEA module, a domain conserved among heavily glycosylated proteins and thought to facilitate binding of glycoproteins to neighboring carbohydrate moieties (64, 65), and cannot be separated by immunoprecipitation with antibodies to the cytoplasmic tail (38, 62). Interestingly, the MUC1 heterodimer is SDS-labile but does not dissociate in the presence of urea or β-mercaptoethanol, indicating that disulfide bond formation is unlikely to stabilize the interaction between the two cleavage products (37, 62). Moreover, studies conducted with the human uterine epithelial cell line, HES, indicate that elevated temperature, high salt, and low pH also fail to disrupt the metabolic cleavage complex (37). Further studies demonstrate that MUC1 is internalized and recycled repeatedly through the trans-Golgi network from the apical cell surface to achieve and maintain a high degree of sialylation (66). A single MUC1 molecule cycling ~10 times through the trans-Golgi network prior to release with the heterodimeric complex presumably surviving the repeated cycling through endosomal compartments (66). Taken together, the apparent stability of the MUC1 heterodimer upon exposure to conditions that might be encountered during recycling or at the cell surface along with the finding that deletion of the region comprising the metabolic cleavage site generates mutant transfectants that are able to release at least as much cell surface MUC1 as the wild-type transfectants (62) implicates a second cleavage event at an alternate stage of processing and argues against dissociation of the MUC1 heterodimer at the cell surface. Nevertheless, Parry et al. (63) have identified an intracellular cleavage site 65 amino acids upstream of the MUC1 transmembrane domain. Although this study did not detect additional amino termini distinct from the metabolic cleavage site, MUC1 was immunoprecipitated from cells with an antibody to a FLAG sequence incorporated into the MUC1 ectodomain. Thus, if a second cleavage occurred, the residual membrane-associated fragment with a potentially different amino terminus would not have been included in the immunoprecipitates produced by the antibody to the FLAG sequence. Therefore, these results do not preclude an additional cleavage event from occurring at an alternate stage of processing.

Because of its extreme resistance to externally added proteases (23), it is unlikely that cell surface MUC1 release is mediated by the actions of an external protease (23, 62). It is
more plausible that there is an endogenous proteolytic system. The studies conducted to date suggest that the MUC1 heterodimer is an extremely stable complex and that dissociation of the intracellular cleavage complex is unlikely, considering that MUC1 mutant transfectants devoid of the intracellular cleavage site would release little, if any, MUC1 if the mechanism of cell surface release were simple dissociation. Collectively, these observations suggest that released MUC1 lacks the cytoplasmic tail (37, 44) and that release is catalyzed by a protease (44, 67). Thus, characterization and identification of activities mediating MUC1 cell surface release are likely to affect our understanding of the biological function of MUC1 in several contexts.

This study explored the hypothesis that MUC1 is released from the cell surface by an enzymatic activity. Based on the protease inhibition profile of the MUC1 sheddase(s), both constitutive and induced MUC1 cell surface release are mediated by a metalloprotease(s). The phorbol ester, PMA, stimulated MUC1 ectodomain release. In most cases where TACE plays a role in ectodomain shedding, it has been in the context of phorbol ester-stimulated shedding (4, 13–15). Serine, cysteine, and aspartyl protease inhibitors had no effect on MUC1 ectodomain release; however, constitutive and PMA-mediated MUC1 shedding were sensitive to the hydroxamate-based metalloprotease inhibitor, TAPI, indicating the involvement of a metalloprotease in constitutive and stimulated shedding of MUC1. Interestingly, TAPI also inhibited constitutive MUC1 shedding from a mammary carcinoma cell line, T47D (data not shown). Furthermore, prodomain removal by a furin-type pro-protein convertase appears to be a prerequisite for metalloproteolytic activity of the ADAMs with a catalytic consensus sequence that has been characterized to date (42, 51, 68, 69). Consistent with this observation, stimulated MUC1 shedding was reduced by a furin inhibitor. Together with the insensitivity to various protease inhibitors and the sensitivity to TAPI and the furin inhibitor, these observations suggest the involvement of a metalloprotease in MUC1 ectodomain release and indicate that MUC1 ectodomain release is not mediated by dissociation of the metabolic cleavage complex in light of the susceptibility of MUC1 release to inhibition by metalloprotease inhibitors.

The endogenous matrix metalloprotease inhibitors, TIMPs, inhibit all of the known matrix metalloproteases (MMPs) to a varying extent (reviewed in Ref. 70). Further studies demonstrate that several membrane-type (MT)-MMPs are effectively inhibited by TIMP-2 and TIMP-3 (71–73). Accordingly, the TIMP inhibition profile of the MUC1 sheddase(s) suggests that...

Fig. 7. Immunolocalization of MUC1 and TACE in tissue sections from the receptive phase human endometrium. A–C, tissue sections were incubated with polyclonal anti-TACE and monoclonal anti-MUC1 (214D4) antibodies followed by incubation with FITC-conjugated goat anti-rabbit and Texas Red-conjugated donkey anti-mouse secondary antibodies. Nuclear (DAPI) staining is shown in the merged images in blue in each case. Luminal epithelial expression of TACE (A), MUC1 (B), and the merged image (C) were visualized by confocal microscopy. D–F, Glandular epithelial expression of TACE (D), MUC1 (E), and the merged image (F) were processed as described in A–C. G–I, the anti-TACE antibody was preincubated with the peptide to which it was generated. Tissue sections then were incubated with the peptide block and the anti-MUC1 antibody followed by FITC-conjugated goat anti-rabbit and Texas Red-conjugated donkey anti-mouse secondary antibodies. TACE peptide block (G), MUC1 (H), and the merged image (I) were processed as described in A–C. J–L, Tissue sections were incubated with the anti-TACE antibody and nonimmune mouse IgG followed by FITC-conjugated goat anti-rabbit and Texas Red-conjugated donkey anti-mouse secondary antibodies. TACE (J), mouse IgG (K), and the merged image (L) were processed as described in A–C.
it is not a known MMP or membrane-type MMP because neither constitutive nor stimulated MUC1 release is inhibited by TIMP-1 or TIMP-2. In addition, ADAM 10/KUZ is probably not responsible for proteolytic processing of MUC1 because it is inhibited by both TIMP-1 and TIMP-3 (53). TIMP-3, however, is highly expressed at the maternal-fetal interface during human implantation, suggesting a regulatory role in trophoblast invasion (74, 75). It also inhibits the metalloproteolytic-dependent shedding of L-selectin (76), syndecan-1 and -4 (77), IL-6R (78), and pro-TNF-α (50). Moreover, TIMP-3 is unique in its ability to inhibit TACE (50, 79) and is the only TIMP found to bind heparan sulfate proteoglycans expressed on the cell surface (80), which may permit co-localization and interaction with cell surface metalloproteases. Thus, the sensitivity of stimulated MUC1 release to TIMP-3 alone and the synthetic metalloprotease inhibitor, TAPI, along with the marked stimulation of MUC1 shedding in response to PMA are consistent with the involvement of an ADAM protease(s), such as TACE, in proteolytic release of MUC1; however, these studies do not exclude additional or alternative proteases.

A role for TACE in the processing of putative substrates comes from analysis of the phenotype of tace<sup>Zn/ΔZn</sup> mice and mice derived from these TACE-deficient mice. Examination of fibroblasts isolated from tace<sup>Zn/ΔZn</sup> mice demonstrates that TGFR-α, L-selectin, p75 TNFR, and β-APP shedding is drastically reduced in comparison to wild-type cells (4, 13). In addition, numerous putative substrates for TACE have been identified utilizing TACE-deficient fibroblasts (14–17, 20, 21). Here, we show that both constitutive and PMA-stimulated MUC1 release by embryonic fibroblasts derived from tace<sup>Zn/ΔZn</sup> mice is completely abolished in comparison to constitutive and stimulated MUC1 shedding from the corresponding EC-4 wild-type cells. Because the tace<sup>Zn/ΔZn</sup> EC-2 cells are an immortalized cell line, the immortalization process may have inactivated a protease(s) expressed in normal cell types. Alternatively, the mutation in the TACE gene may have affected a linked gene; thus, the defect in MUC1 shedding may not directly be caused by a loss of TACE. However, co-transfection of TACE and MUC1 cDNA into the TACE-deficient EC-2 cells completely restored constitutive as well as PMA-stimulated MUC1 release. In addition, primary embryonic fibroblasts devoid of ADAMs 9, 12, and 15 shed MUC1 normally when compared with their wild-type counterparts, eliminating these ADAMs as potential MUC1 sheddases in this system. Collectively, these results present compelling evidence for TACE as a MUC1 sheddase. Nevertheless, further studies will be necessary to address whether TACE mediates MUC1 ectodomain release at the same site in the HES cells and in wild-type and TACE-deficient fibroblasts.

Previous findings in rabbits indicate a hormonally and blastocyst-dependent increase in ADAM 9 expression and MUC1 loss at regions of cell-cell adhesion and fusion in the uterus during implantation (32), suggesting a crucial role for ADAM 9 in this species. Clearance of MUC1 from the surface of human uterine epithelial cells also appears to be a necessary prerequisite for the creation of an environment conducive to blastocyst attachment. In conjunction with the identification of MUC1 as a TACE substrate and the establishment of an interaction between MUC1 and TACE in HES cells in vitro, localization of both MUC1 and TACE was observed in vivo in luminal and glandular epithelial cells from the receptive phase human endometrium. MUC1 is predominantly found on the luminal and glandular epithelial cell surface on microvilli with only modest intracellular localization, whereas TACE is primarily expressed intracellularly with moderate cell surface expression apparent on luminal and glandular epithelial cells.

The relatively minor degree of intracellular co-localization of MUC1 and TACE suggests that, if TACE is a MUC1 sheddase in vitro, at least a portion of MUC1 cleavage probably occurs in an intracellular compartment. Consistent with this finding, various catalytic ADAMs, including TACE, are localized mainly in intracellular compartments and therefore might be active intracellularly as well as on the cell surface (42). However, in vitro cleavage studies indicate that a peptide corresponding to intracellular metabolic cleavage site of MUC1 is not a putative substrate for TACE nor are other peptides corresponding to potential cleavage sites within the SEA module of MUC1 (data not shown), suggesting that TACE does not mediate cleavage of MUC1 at the metabolic cleavage site or at additional potential cleavage sites within the SEA module. These putative TACE substrates, however, may not be cleaved well in vitro, implicating associations distal to the cleavage site as necessary in the regulation of MUC1 shedding. Correlatively, the EGF domain of L-selectin appears to serve as a protease recognition motif for phorbol ester-stimulated shedding of this molecule (43). It also has been suggested that interactions with the cytoskeleton might modulate proteolytic activity by shifting the localization of proteases to membrane domains proximal to their substrates following activation (2). In this regard, during the period of receptivity in humans and several other species, the fluidity of the plasma membrane increases and microvilli on the apical surface of the luminal epithelium become shorter, more blunted, and irregular (82, 83). Thus, the uterine morphological changes that accompany generation of a receptive uterine state may temporarily redistribute MUC1 to a membrane domain where it is more susceptible to cleavage by TACE. Alternatively, a potential physiologically relevant stimulus of MUC1 shedding might trigger redistribution of TACE and/or MUC1 and thereby enhance MUC1 shedding.

In conclusion, the current studies provide the initial characterization of TACE as a candidate MUC1 sheddase in a model of human uterine epithelia. We demonstrate that MUC1 shedding is stimulated by treatment with a phorbol ester. Moreover, based on the inhibition profile of the sheddase(s), both constitutive and stimulated activities appear to be mediated by the same or a similar protease. The finding that MUC1 and TACE form stable associations implies that complexation may exist in uterine epithelia that are poised for rapid cleavage of MUC1. This may be of importance in human implantation, where local cleavage of the MUC1 barrier by the blastocyst is likely. It remains possible that additional proteases may cleave MUC1 in vivo. Thus, determining whether TACE or a related ADAM(s) mediates cell surface release of MUC1 provides a new venue for the study of MUC1 biology and potential therapeutic targets for MUC1-dependent processes.

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