Tumor Necrosis Factor-α-converting Enzyme (TACE/ADAM-17) Mediates the Ectodomain Cleavage of Intercellular Adhesion Molecule-1 (ICAM-1)*

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Ectodomain shedding has emerged as an important regulatory step in the function of transmembrane proteins. Intercellular adhesion molecule-1 (ICAM-1), an adhesion receptor that mediates inflammatory and immune responses, undergoes shedding in the presence of inflammatory mediators and phorbol 12-myristate 13-acetate (PMA). The shedding of ICAM-1 in ICAM-1-transfected 293 cells upon PMA stimulation and in endothelial cells upon tumor necrosis factor-α stimulation was blocked by metalloproteinase inhibitors, whereas serine protease inhibitors were ineffective. p-Aminophenylmercuric acetate, a mercuric compound that is known to activate matrix metalloproteinases, up-regulated ICAM-1 shedding. TIMP-3 (but not TIMP-1 or -2) effectively blocked cleavage. This profile suggests the involvement of the ADAM family of proteases in the cleavage of ICAM-1. The introduction of enzymatically active tumor necrosis factor-α-converting enzyme (TACE) into ICAM-1-expressing cells up-regulated cleavage. Small interfering RNA directed against TACE blocked ICAM-1 cleavage. ICAM-1 transfected into TACE−/− fibroblasts did not show increased shedding over constitutive levels in the presence of PMA, whereas cleavage did occur in ICAM-1-transfected TACE+/+ cells. These results indicate that ICAM-1 shedding is mediated by TACE. Blocking the shedding of ICAM-1 altered the cell adhesive function, as ICAM-1-mediated cell adhesion was up-regulated in the presence of TACE small interfering RNA and TIMP-3, but not TIMP-1. However, cleavage was found to occur at multiple sites within the stalk domain of ICAM-1, and numerous point mutations within the region did not affect cleavage, indicating that TACE-mediated cleavage of ICAM-1 may not be sequence-specific.

Intercellular adhesion molecule-1 (ICAM-1), a cell adhesion molecule expressed in various cell types, plays a key role in mediating the inflammatory and immune responses (1). It functions as a co-stimulatory molecule during antigen presentation to T cells. ICAM-1 interaction with leukocyte integrins LFA-1 and Mac-1 promotes firm adhesion of leukocytes and their transmigration to the sites of inflammation (1–3). ICAM-1 consists of five Ig-like domains, a single transmembrane domain, and a short cytoplasmic tail. ICAM-1 is shed as soluble ICAM-1 (sICAM-1) in blood and other biological fluids. Elevated plasma levels of sICAM-1 have been reported in inflammatory, neoplastic, autoimmune, and vascular diseases (4), and sICAM-1 is utilized as a marker of inflammation and a predictor of prognosis (5, 6). Multiple signaling pathways regulate the shedding of ICAM-1 in 293 cells transfected with ICAM-1 (293ICAM-1), including mitogen-activated protein kinase and phosphatidylinositol 3-kinases (7). How these signaling cascades mediate ICAM-1 release is currently unknown. Previous reports indicate that ICAM-1 is a substrate for matrix metalloprotease (MMP)-9 (8) as well as human leukocyte elastase and cathepsin G (9, 10).

Ectodomain shedding is an important regulatory mechanism in the function of membrane-bound cell-surface molecules (reviewed in Refs. 11 and 12). Cytokines and their receptors, growth factors, ectoenzymes, adhesion molecules, and proteoglycans undergo shedding (13–16). The most widely studied inducer of shedding is phorbol 12-myristate 13-acetate (PMA), which activates protein kinase C (16, 18–20). Calcium ionophores, cytokines, growth factors, and chemotactic peptides also induce shedding (22). The majority of the shedding events are mediated by the zinc-dependent metalloproteinases of the metzincin family, which includes MMPs and proteases containing a disintegrin and metalloproteinase (ADAM) domain. Tissue inhibitors of metalloproteinases (TIMPs) regulate the activity of MMPs and some ADAMs. There are four distinct TIMPs. TIMP-1–4 inhibit a wide range of MMPs, whereas TIMP-3 is also an inhibitor of ADAM-17. ADAM-10 is inhibited by TIMP-1 and -3, whereas ADAM-8 and -9 are very poorly inhibited by, and are unlikely to be physiologically regulated by, TIMPs. To date, no ADAM has been shown to be inhibited by TIMP-2. ADAMs are type I transmembrane glycoproteins; and in addition to their proteolytic activity, they also mediate cell adhesion. Tumor necrosis factor-α (TNFα)-converting enzyme (TACE) plays a central role in ectodomain shedding (16). Cell lines derived from TACE-deficient mice (TACE−/−) verify that TACE is involved in the PMA-induced shedding of a number of structurally and functionally diverse proteins, including pro-TNFα, l-selectin, β-amyloid precursor protein, transforming growth factor-α, heparin-binding epidermal growth factor (EGF), and vascular cell adhesion molecule-1 (VCAM-1) (14, 17–21), suggesting a common shedding mechanism regulated by a protein kinase C-TACE axis. Mice lacking TACE have a severe phenotypic defect that resembles EGF receptor- and transforming growth factor-α-deficient mice (23), suggesting an essential role for TACE in development and emphasizing the impor...
tance of ectodomain shedding in vivo (14). Our results now provide evidence that TACE (ADAM-17) mediates ICAM-1 shedding. The shedding of ICAM-1 reduced the adhesive capacity of the cells. The TACE cleavage site in ICAM-1 is not sequence-specific, but appears to be nonselective, as has been reported for other TACE substrates.

**EXPERIMENTAL PROCEDURES**

Reagents—EDTA and EGTA were purchased from J. T. Baker Inc. Phosphoramidon and MMP inhibitor III (MMPI-III) were from Calbiochem; soybean trypsin inhibitor was from Invitrogen (Paisley, UK); and BB-94 was from British Biotech (Oxford, UK). TNFα protease inhibitor-1 (TAPI-1; Peptide International Inc., Louisville, KY) was a kind gift from Drs. R. Gray and A. Spatola. TIMP-1, -2, and -3 were prepared as described previously (24–26). PMA, p-aminophenylmercuric acetate (APMA), phenylmethylsulfonyl fluoride, and trichloroacetic acid were obtained from Sigma. The BCA protein assay kit was from Pierce, and the enhanced chemiluminescence (ECL) detection reagents were from Amersham Biosciences. Polyclonal antibody R98 (directed against the ICAM-1 cytoplasmic sequence, A9KGTMPKNTQAPTP) was developed in our laboratory (7), as were antibodies against the ICAM-1 ectodomain (R686 and R803). Monoclonal antibody LB-2 (raised against the ICAM-1 ectodomain) was from BD Biosciences. Monoclonal antibodies against integrin subunits α1, α5, and β3 were purchased from BioLegend (San Diego, CA). Horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad. The antibody to TACE was obtained from QED Bioscience Inc. The enzyme-linked immunosorbent assay kit for the detection of human sICAM-1 was obtained from Pierce, and the non-stimulated and stimulated lysates were collected and stored at 4°C for analysis.

**Transfection—**Human wild-type TIMP-3 cDNA was a gift from Dr. D. R. Edwards (University of East Anglia, Norwich, UK). Human mutant TIMP-3(C15) cDNA (a gift from Dr. M. Bond, Bristol Heart Institute, Bristol, UK) (29) was subcloned into pEGFP-N1 (Clontech), replacing the enhanced green fluorescent protein cDNA (30). Mouse TACE cDNA in pMOS was a gift from Celltech Chiroscience (Bothell, WA) (28). The TACE cDNA was subcloned into pcDNA3.1/Zeo+ (Invitrogen), and a FLAG peptide was inserted immediately preceding the proposed proprotein convertase cleavage site using standard PCR technology and sequenced. To transiently express human wild-type TIMP-3, human mutant TIMP-3(C15), or mouse TACE in 293ICAM-1 cells, cells were grown to 60–80% confluence on 12-well plates and transfected with 0.5–2.0 μg of the appropriate DNA using Lipofectamine Plus™ (Invitrogen). Briefly, for each well in transfection, DNA was diluted in 25 μl of DMEM without serum and containing 5 μl of Lipofectamine Plus reagent. This mixture was incubated for 15 min at room temperature. 2 μl of Lipofectamine reagent was mixed into 25 μl of DMEM without serum. The diluted DNA and Lipofectamine reagent were mixed and incubated for 15 min at room temperature to allow DNA-liposome complexes to form. While complexes were being formed, the medium on the cells was replaced with 0.4 ml of DMEM without serum. For each transfection, 50 μl of the DNA/liposome mixture was overlaid and mixed gently. The plate was incubated at 37°C in a CO2 incubator. After 5 h of incubation, the medium was replaced with medium containing serum and incubated further. Cell lysates were assayed for transient gene expression 48 h after transfection.

**Small Interfering RNA (siRNA) Transfection—**TACE siRNA was purchased from Dharmacon (Lafayette, CO). Transfection of siRNA was performed using GenePORTER transfection reagent (Gene Therapy Systems, Inc., San Diego). Briefly, 293ICAM-1 cells/ECs were grown to 60–80% confluence on 24-well plates. 5 μl of the transfection reagent was diluted in 0.125 ml of DMEM. 2 μg of siRNA was also separately diluted in 0.125 ml of DMEM. They were mixed together and incubated at room temperature for 45 min. The mixture was overlaid on the cells and incubated at 37°C in a CO2 incubator. After 2–5 h, the overlaid siRNA/reagent mixture was diluted by adding 0.25 ml of complete medium containing 5% FCS and further incubated for 24–48 h. The transfected 293 cells were stimulated with PMA (3 μM) for 3 h. The transfected ECs were stimulated with TNFα (10 ng/ml) for 16 h. Both the non-stimulated and stimulated lysates were collected and stored at 4°C for analysis.

**Transfection of Immortalized Mouse EC2 and EC4 Fibroblasts with Human ICAM-1 cDNA and Analysis of ICAM Shedding—**EC2 and EC4 fibroblasts were seeded onto 6-well dishes in DMEM and transfected for 16 h with pcDNA3.1 alone or with human ICAM-1 cDNA using FuGENE 6 (Roche Diagnostics) according to the manufacturer’s instructions. The medium was then replaced with 1 ml of prewarmed fresh medium prior to incubation with or without BB-94 for 30 min at a final concentration of 10 μM. Cells were incubated with or without 3 μM PMA for 6 h. The medium was then harvested and subjected to centrifugation in a microcentrifuge for 5 min at 16,000 × g before analysis by enzyme-linked immunosorbent assay. EC2 and EC4 cell lines were found to transfect at different efficiencies. To derive a correction factor, separate wells of each cell line were transfected with 0.5 μg of pEGFP-N1 in duplicate, and the enhanced green fluorescent protein fluorescence of the cells was quantitated using a Tecan Spectrafluor Plus plate reader with the appropriate filters.

**Analysis of ICAM-1 Shedding and Inhibitor Assays—**Treatment of 293ICAM-1 cells was conducted under serum deprivation conditions. The medium was replaced with DMEM containing 1% FCS 1 h before the experiment. Media substitution caused little cell death, as cells remained firmly attached to the plate surface. Inhibitors were added at the indicated concentrations and incubated for 3 h at 37°C. After incubation with PMA (3 μM), cells were incubated for an additional 3 h at 37°C. ECs were supplemented with fresh medium containing 10% FCS 1 h before the experiment. Cells were stimulated with TNFα and incubated for 6 h, after which time inhibitors were added as indicated, and cells were incubated for an additional 18 h at 37°C. After treatment, cell morphology and degree of adhesion were assessed, and cell viability was estimated using the trypan blue exclusion assay. Drug concentrations were maintained in a range that did not affect cell viability. After treatment, cells were rinsed with DMEM and then lysed with ice-cold lysis buffer (10 mM Tris (pH 7.5), 5 mM EDTA, 50 mM NaCl, 0.5% Triton X-100, 0.1% SDS, and 1% Nonidet P-40). Protein concentration was estimated using the BCA protein assay kit.
measured using the BCA kit. Aliquots containing equal amounts of total protein were analyzed by 16.5% Tricine/SDS-PAGE (32). Proteins were transferred to polyvinylidene difluoride membranes and immuno-blotted with a primary antibody, followed by a horseradish peroxidase-conjugated secondary antibody. Blots were developed using the ECL detection system. The intensity of the signals on the autoradiograms was quantitated using UN-SCAN-IT software. The signal intensity in the absence of inhibitors was taken as 100%, and the degree of inhibition was expressed accordingly as a percentage. Each data point represents the results of at least three separate experiments (means ± S.E.).

Adhesion Assay—Adhesion of THP-1 cells to ECs was performed as described previously (7). Briefly, ECs grown on 24-well culture plates were stimulated with TNFα and treated with inhibitors. Monocytic THP-1 cells were gently added to the EC monolayer and incubated for 1 h at 37 °C. Nonadherent cells were removed. Adherent cells were stained with 0.25% rose Bengal. The excess stain was removed, and cells were washed. The stain was extracted with ethanol/Dulbecco’s phosphate-buffered saline, and the absorbance was measured at a wavelength of 570 nm. The absorbance of the non-stimulated ECs in the absence of THP-1 cells was taken as the background and subtracted from the other values. Each data point represents the results of three separate experiments. Statistical analysis was performed with Student’s t test. Differences were considered significant when p ≤ 0.05.

Cleavage Site Analysis—The cleavage site was assessed by molecular mass analysis using the Micromass instrumentation that utilizes electrospray ionization time-of-flight mass spectrometry. The ~7-kDa fragment of ICAM-1 was eluted out of gel, desalted, and subjected to electrospray ionization mass spectrometry. Several point mutations in the “stalk” region of wild-type ICAM-1 on pcDNA3.1 (7, 27), viz. R433A, R433K, R431A, E438A, R451A, N446A, R441A/K442A, were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). 293 cells were transiently transfected with each of the above mutations, and PMA-induced cleavage was examined and compared with that of wild-type ICAM-1 as described above.

RESULTS

MMPs Regulate ICAM-1 Shedding—Cleavage was monitored utilizing an antibody directed against the cytoplasmic sequence of ICAM-1, which recognizes both full-length ICAM-1 (~93 kDa) and a cytoplasmic fragment (~7 kDa) remaining after ectodomain cleavage. In 293 ICAM-1 cells, there is a certain level of constitutive shedding, which is increased by 2–3-fold upon stimulation with PMA (7). The detection of the membrane-bound 7-kDa fragment in cell lysates corresponds to the appearance of sICAM-1 in the culture medium (7). Protease inhibitors of different classes were utilized to identify enzyme(s) responsible for the shedding of ICAM-1.

EDTA and EGTA—Metal chelators of divalent cations dose-dependently inhibited the cleavage of ICAM-1 in PMA-stimulated 293 cells and in TNFα-stimulated ECs, indicating that a metal-dependent or metal-containing protease(s) is involved in the release of ICAM-1. EDTA and EGTA at 5 mM inhibited cleavage by >80% in 293 ICAM-1, and by >90% in ECs (Table 1). We observed no significant effect with soybean trypsin inhibitor and phenylmethylsulfonyl fluoride, thus indicating that serine proteases are unlikely to be involved in ICAM-1 shedding. However, three different inhibitors of MMPs were effective in blocking cleavage. Phosphoramidon, the least effective of the three reagents, displayed inhibitory activity at higher concentrations and at 80 μM caused 72% inhibition in 293 ICAM-1 cells, whereas the degree of inhibition was ~55% in ECs (Table 1). Phosphoramidon is not a potent inhibitor of the metalloproteases, and its ineffectiveness is to be expected if an ADAM or a membrane-type MMP is involved in ICAM-1 shedding. TAPI-1, known to inhibit conventional MMPs and ADAMs, at 25 μM caused >90% inhibition in 293 ICAM-1 cells. TAPI-1 was very effective even at 10 μM. In ECs, the degree of inhibition was 65% at 75 μM. MMPI-III (effective against MMP-1, -2, -3, -7, and -13) at 100 μM blocked cleavage by >80% in both cell types (Table 1). As the S-guideline is not known to contribute significantly to an inhibitor’s interaction with TACE, MMPI-III can be considered similar in properties to Ro-31-9790. MMPI-III has the same specificity for MMP-1, -2, and -3 as Ro-31-9790, and it is likely that its IC50 for TACE will be approximately similar, i.e. 30–50 μM (Calbiochem) (31), in agreement with its potency in inhibiting ICAM-1 shedding. These inhibitors had very similar effects on the constitutive shedding of ICAM-1 (data not shown).

The mercuric compound APMA is known to activate in vitro MMPs as well as ADAMs (20). As shown in Fig. 1A, APMA up-regulated the cleavage of ICAM-1 by >2-fold with the corresponding production of sICAM-1 in the culture medium in a dose- and time-dependent manner. Interestingly, the cytoplasmic fragment generated upon stimulation with APMA migrated at the same ~7-kDa level as the one induced by PMA stimulation. sICAM-1 detected in the culture medium after APMA and PMA treatment was also indistinguishable (data not shown). Therefore, a protease(s) induced by these two compounds causes cleavage at a common site or at sites in close proximity. When APMA and PMA were applied simultaneously, no further increase in the amount of ICAM-1 shedding was observed (data not shown). These results indicate that ICAM-1 shedding is mediated by a member of the MMP-like family of proteases.

TABLE 1

| Inhibitors       | Inhibition |
|------------------|------------|
|                  | ECs 293 ICAM-1 cells |
| EDTA (5 mM)      | 95         |
| EGTA (5 mM)      | 95         |
| Phosphoramidon (80 μM) | 55         |
| TAPI-1 (75 μM)   | 65         |
| MMPI-III (100 μM) | 82         |
| SBTI* (200 μg/ml) | 0          |
| PMSF* (2 mM)     | 7          |

* SBTI, soybean trypsin inhibitor.

PMSF, phenylmethylsulfonyl fluoride.

The differing protease specificities of TIMPs permit the determination of whether MMPs or ADAMs are implicated in ectodomain processing (33, 34). The effects of application of purified TIMP-1, -2, and -3 at 500 nM to stimulated ECs and 293 ICAM-1 cells were assessed. TIMP-1 had no effect on ICAM-1 shedding. TIMP-2 caused minimal inhibition, whereas TIMP-3 caused >50% inhibition in both cell types (Fig. 1B). At the concentrations used in our study, TIMP-1 and -2 inhibit almost all known MMPs, and TIMP-1 would be expected to inhibit ADAM-10 (24). Transfection of TIMP-3 into 293 ICAM-1 cells resulted in the inhibition of ICAM-1 shedding by >50% (Fig. 1C). TIMP-3 is active against TACE (31), whereas TIMP-1 and, to a lesser extent, TIMP-3 are effective against ADAM-10. Both ADAM-10 and -17 are present in ECs,

FEBRUARY 10, 2006 • VOLUME 281 • NUMBER 6 JOURNAL OF BIOLOGICAL CHEMISTRY 3159
FIGURE 1. A, effect of APMA. Serum-depleted 293<sub>ICAM-1</sub> cells were incubated with APMA at the indicated concentrations for 1 h. Cells were lysed and subjected to 16.5% Tricine/SDS-PAGE and Western blotting with antibody R98 (left panels). The cell culture medium was trichloroacetic acid (TCA)-precipitated and subjected to 10% SDS-PAGE and Western blotting using antibody R686 (directed against the ectodomain of ICAM-1) (right panels). WT, wild-type. B, effect of TIMP-1, -2, and -3. Serum-depleted ECs were stimulated with TNFα (10 ng/ml) for 6 h (left); TIMP-1, -2, and -3 were added at 500 nM and incubated for an additional 18 h at 37 °C. Serum-depleted 293<sub>ICAM-1</sub> cells were stimulated with PMA (3 μM) for 3 h at 37 °C (right); TIMP-1, -2, and -3 were added at 500 nM and incubated for an additional 3 h. Cells were lysed and subjected to 16.5% Tricine/SDS-PAGE and Western blotting with antibody R98. C, effect of TIMP-3 transfection. 293<sub>ICAM-1</sub> cells were transiently transfected with TIMP-3 cDNA. Following transfection, cells were stimulated with PMA (3 μM) for 3 h. Cells were lysed at the indicated time points; lysates were subjected to SDS-PAGE and immunoblotting; and the results were analyzed as described in the legend to Table 1. The data shown are representative of at least three independent experiments.
but only inhibition by TIMP-3 is observed. This strongly indicates that TACE is involved in the shedding of ICAM-1. The effectiveness of TAPI-1 in blocking ICAM-1 cleavage (Table 1) also indicates a possible role for TACE in ICAM-1 shedding.

TACE (ADAM-17) Mediates ICAM-1 Shedding—To determine whether TACE mediates ICAM-1 shedding, TACE was transected into ICAM-1-expressing 293 cells, and cells were stimulated with PMA. TACE increased the amount of ICAM-1 cleavage by 100% (Fig. 2A). These results indicate that TACE is a potential sheddase mediating ICAM-1 release. This was further verified by the application of siRNA directed against TACE. siRNA was transfected into 293 cells; cells were stimulated with PMA; and ICAM-1 cleavage was assessed using antibody R98. We found that siRNA blocked ICAM-1 release in 293 cells. As shown in Fig. 2B (upper panel), the PMA-stimulated shedding of ICAM-1 was inhibited by 57%. In ECs, TACE siRNA inhibited ICAM-1 shedding by 74% (Fig. 2C, upper panel). Moreover, when we analyzed the effectiveness of the siRNA, we found that siRNA completely blocked TACE expression in both cell types (Fig. 2, B and C, lower panels). Interestingly, although siRNA was very effective in blocking the expression of TACE (>95% inhibition in both cell types), the cleavage of ICAM-1 was not completely inhibited, indicating a possible role for other proteases in the shedding process.

To confirm the involvement of TACE in shedding, fibroblasts derived from TACE knockout mice were transfected with ICAM-1, and cleavage was assessed by measuring the concentration of sICAM-1 in the culture medium by an enzyme-linked immunosorbent assay specific for human ICAM-1. As shown in Fig. 3, both constitutive and PMA-stimulated shedding were deficient in TACE−/− cells. The constitutive shedding of ICAM-1 was inhibited by 52%, whereas PMA-stimulated release was inhibited by 73%. Both constitutive and stimulated shedding were sensitive to BB-94 (33 and 71% inhibition, respectively), a broad-spectrum and potent inhibitor of MMPs and ADAMs. These results confirm that TACE is required for both constitutive and PMA-induced ICAM-1 release.

Functional Implication of ICAM-1 Shedding—We studied the effect of TACE inhibition on cell adhesion mediated through ICAM-1 (7). In the assay performed, THP-1 cell adhesion to TNFα-stimulated ECs is a result of ligation of ICAM-1 on ECs to β2 integrins on THP-1 cells. Upon preincubation with ECs at 20 μg/ml for 1 h, anti-ICAM-1 antibody LB-2 blocked THP-1 cell adhesion by 80%, whereas upon preincubation with THP-1 cells, antibodies against integrin subunits αI, and αI,M (20 μg/ml) blocked adhesion by 80%. Antibody against β2 integrin abolished 95% of THP-1 cell adhesion, thus indicating the specificity of the adhesive interaction.

ECs were treated with TIMP and TACE siRNA as shown in Figs. 1B and 2C, respectively. Monocytic THP-1 cells were allowed to adhere to ECs to assess whether inhibition of cleavage affects ICAM-1 adhesion function. As shown in Fig. 4A, TIMP-3 dramatically (by 91%) enhanced adhesion of THP-1 cells to ECs. Similarly, TACE siRNA up-regulated adhesion by 89% (Fig. 4B). These results indicate that TACE-mediated
ICAM-1 shedding reduces functionally intact ICAM-1 and consequently reduces EC adhesive function with leukocytes.

Identification of the ICAM-1 Cleavage Site—Analysis of previous reports regarding the TACE cleavage site in several different substrates demonstrated a preference for the site with a hydrophobic residue at the membrane-proximal site (35–38). Mass spectrometric analyses of the PMA-induced 7-kDa fragment of ICAM-1 from 293 cells indicated heterogeneous fragmentation following Arg⁴³¹, Arg⁴³³, and Lys⁴⁴², thus suggesting nonselective cleavage, as has been noted previously for other proteins (36, 37). For further verification of the cleavage specificity of ICAM-1, we introduced several point mutations into the membrane-proximal region of ICAM-1, including those identified by mass spectrometry, to examine whether cleavage would be hampered through mutations (Fig. 5). However, none of the mutations prevented the shedding of ICAM-1. We noted the appearance of a doublet in R431A, indicating that cleavage also occurs at the alternative site. These results indicate that the ectodomain cleavage site of ICAM-1 is nonsequence-specific and occurs in a nonselective manner.

DISCUSSION

This study provides evidence that ICAM-1 shedding is mediated by TACE (ADAM-17). This evidence was developed initially using protease inhibitors, which suggested that the candidate sheddase was a metal-dependent protease belonging to the large family of MMPs or ADAMs (Table 1). Because TIMP-1 and -2 were ineffective in blocking ICAM-1 cleavage in both 293 cells and ECs (Fig. 1B), the role for classical MMPs in ICAM-1 shedding was eliminated. The indication that TIMP-3 specifically blocked cleavage suggested a role for ADAMs in the shedding process (Fig. 1B and C). TIMP-3 is an effective inhibitor of TACE (31). We focused on TACE, as this protease is present in 293 cells and ECs and has been involved in the PMA-induced cleavage of other proteins (16–21). Transfection of TACE into ICAM-1-expressing cells augmented ICAM-1 cleavage (Fig. 2A). The introduction of siRNA (to specifically inactivate TACE) into ICAM-1-expressing cells resulted in reduction of ICAM-1 cleavage (Fig. 2A). Further evidence was obtained using fibroblasts from TACE-deficient mice. ICAM-1-transfected TACE⁻/⁻ fibroblasts were resistant to cleavage upon stimulation with PMA, whereas TACE⁺/⁺ cells processed ICAM-1 in a normal manner (Fig. 3). Therefore, ICAM-1 belongs to the growing number of transmembrane proteins representing substrates of TACE.

TACE has been recognized as an important mediator of regulated ectodomain shedding. Previous studies (39) identified the preferential site for TACE cleavage as Ala ↓ Val. However, a more recent study demonstrated efficient shedding by TACE at divergent cleavage sites (40). There is one Ala-Val pair located within the transmembrane domain of ICAM-1 and none within the juxtamembrane stalk domain, where the majority of the shedding events are known to occur (11). However, apart from the Ala ↓ Val site in TNFα (35) and transforming growth factor-α (36), TACE cleaves at Lys-Ser in L-selectin (37) and amphiregulin (36), at Arg ↓ Ser in angiotensin-converting enzyme (ACE) (38), and at Pro ↓ Val in heparin-binding EGF (36). Therefore, TACE seems to have a preference for the site with a hydrophobic residue at the membrane-proximal side. We initially attempted to identify the site of ICAM-1 cleavage by mass spectrometric analysis. However, this indicated multiple cleavage sites. We therefore resorted to introducing point mutations into the membrane-proximal region of ICAM-1. None of the mutations prevented the shedding of ICAM-1 (Fig. 5). Similar results from several earlier reports have shown that mutations close to the cleavage site have very little effect on the processing of different TACE substrates (22, 41, 42).

There appears to be several structural characteristics of a substrate that are important for its recognition by an enzyme, including sequence, stalk length, membrane distance, and features of the distal ectodomain, as well as the tertiary structure of the enzyme itself (16). There is considerable variability among different TACE substrates as to which features are functional in individual molecules. Stalk sequence appears to be important in many instances, as substitution of stalk sequences from shed proteins with those from unshed proteins confers shedding in the
latter group (41, 43). In the case of TNFα, the cleavage site is sufficient for its cleavage by TACE, as cleavage still occurs even in the absence of the ectodomain (35). With ACE, conservation of the amino acid sequence is not essential for shedding. The critical parameter is the conformation of the stalk rather than the sequence (44, 45). The length of the stalk or the distance from the membrane appears to be important in the cleavage of TNFα, growth hormone receptor, L-selectin, and ACE, presumably reflecting an essential role for the accessibility of the cleavage site by the enzyme. Deletion of several residues in the membrane-proximal domain, even with preservation of the cleavage site, prevents cleavage (35, 42). In the case of the Kit ligand, in chimeric containing repeated stalk sequences, cleavage occurs only in the membrane-proximal cleavage site, emphasizing the importance of the distance from the membrane (46). The shedding of EGF receptor family members (transforming growth factor-β, amphiregulin, and heparin-binding EGF) is determined by stalk length and target bond sequence (36). Several studies using constructs combining shed and unshed molecules underline the importance of structural features of the distal ectodomain for efficient cleavage (42, 45). Even with the same stalk sequence, testis ACE is cleaved more efficiently, probably because of a distal structural motif that is recognized by the enzyme (47). Interestingly, the cleavage of ACE is dramatically increased upon mutation in close proximity to the cleavage site. This could perhaps be due to optimal positioning of the ectodomain loop for cleavage to occur (38). The catalytic domain of TACE shows distinctive surface projections, which may play a role in protease-substrate recognition (48) and may in fact contact site(s) remote from the actual cleavage site. Although these factors may be functional in ICAM-1 shedding site recognition by TACE, cleavage itself appears to occur in a nonselective manner.

Recently, MMP-9 was implicated in ICAM-1 shedding in the human promyelocytic leukemia cell line HL-60, and this process modified tumor cell resistance to natural killer cell-mediated cytotoxicity (8). In this study, MMP-9 was shown to co-localize with ICAM-1 and induced its cleavage in vitro, which was blocked by the MMP-specific inhibitors. ICAM-1 is also susceptible to cleavage by elastase and cathepsin G (9). Alternative splicing of ICAM-1 modifies its susceptibility to cleavage by these enzymes, with the common form being most resistant to cleavage, and may thus modify immune interactions mediated through ICAM-1 (10). It is conceivable that different proteases may become activated under different conditions, with ICAM-1 shedding being a substrate for more than one enzyme in different cell lines.

Several shed molecules can be cleaved by more than one enzyme in different cell lines and under different physiological conditions. Amyloid precursor protein shedding is mediated by ADAM-10/9 in 293 cells (49) and by TACE in Chinese hamster ovary cells (50); similarly, heparin-binding EGF cleavage is mediated ADAM-9 in Vero cells (51) and by TACE in Chinese hamster ovary cells (20). TNFα can be cleaved by ADAM-10 (52) and MMP-7 (53) in addition to TACE. The reverse is also true: some enzymes can cleave more than one substrate, and a good example is TACE. Up-regulation of the shedding rate by PMA is considered to be a common feature of the shedding events mediated by TACE. In our cell system, cleavage was up-regulated upon stimulation with PMA (7). Similarly, in other work, ICAM-1 shedding was enhanced by PMA (8). In contrast, ICAM-1 release was not found to be affected by PMA in NIH 3T3 cells, whereas VCAM-1 shedding was significantly up-regulated (21). Thus, different cell types may vary considerably in their shedding characteristics. TACE was found to be involved in both constitutive and PMA-induced shedding of ICAM-1, yet it mediated only PMA-stimulated VCAM-1 shedding (21). We have previously shown that signaling mechanisms mediating ICAM-1 shedding in different cell types activated by different agonists have common as well as some unique features (7). Thus, the involvement of different proteases is quite possible. As ICAM-1 is expressed in diverse cell types, the differential regulation may be designed to target only selected cell populations under different conditions.

The diversity of ICAM-1 architecture and presentation on cell surface, including the ability to dimerize, may affect its susceptibility to proteolysis by the different agents. Recently, ICAM-1 was shown to shed as a dimer in pleural spaces during inflammation (54). It is not yet known how the protease and its substrate are brought together. It is believed that the shedding of transmembrane proteins by ADAMs requires the presence of the membrane-bound enzyme and its substrate in cis-orientation in the membrane (13). The physiological role of ICAM-1 shedding is currently unknown. The processing of cell-surface molecules by ectodomain shedding may represent an important regulatory mechanism for cellular function (11). Mechanistically, removal of cell-surface ICAM-1 prevents ICAM-1-mediated events (e.g. leukocyte adhesion/transmigration and fibrinogen deposition) and limits the extent of local inflammation. Because sICAM-1 is capable of ligand interaction, it may decrease ICAM-1-mediated interactions on the vasculature (55). Our results indicate that shedding interferes with ICAM-1 function and affects intercellular adhesion mediated through ICAM-1 (Fig. 4, A and B). sICAM-1 induces angiogenesis and is involved in the production of cytokines (56). Other studies demonstrate a unique role played by the membrane-anchored fragments generated by ectodomain cleavage: a membrane-anchored cytoplasmic fragment of CD44 is further cleaved, resulting in the release of a smaller cytoplasmic fragment, which translocates to the nucleus and functions as a transcriptional activator (57). Thus, ectodomain shedding may affect cellular functions in diverse ways.

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