Selecting and specific regulation of ectodomain shedding of angiotensin-converting enzyme 2 by tumor necrosis factor α-converting enzyme

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Iwata M, Silva Enciso J, Greenberg BH. Selective and specific regulation of ectodomain shedding of angiotensin-converting enzyme 2 by tumor necrosis factor α-converting enzyme. Am J Physiol Cell Physiol 297: C1318–C1329, 2009. First published September 16, 2009; doi:10.1152/ajpcell.00036.2009.—Angiotensin-converting enzyme 2 (ACE2) is a newly identified regulator of the renin-angiotensin system. This type I membrane-anchored protein has a catalytically active ectodomain that undergoes shedding. Tumor necrosis factor α-converting enzyme (TACE) has been shown to be involved in ACE2 shedding. Although pathophysiological significance of ACE2 shedding has been suggested, regulation of this process by TACE is not clearly defined. We characterized TACE-mediated constitutive ectodomain shedding of ACE2 using wild-type Chinese Hamster Ovary (WT-CHO), the TACE-mutant M2 (M2-CHO) cells, and EC-4 and EC-2 cells that are fibroblasts from wild-type and TACE-null mice, respectively. ACE2 was constitutively cleaved to release two distinct major soluble forms. The deglycosylated molecular masses of the larger (LSF) and smaller soluble form (SSF) were ~80 and 70 kDa, respectively. These forms had equivalent enzyme activities. Reduced shedding for the LSF from M2-CHO and EC-2 cells when compared with WT-CHO and EC-4 cells, respectively, was noted. TACE reconstitution in EC-2 cells expressing ACE2 resulted in increase in LSF but not SSF release, demonstrating a main role of TACE in LSF release and distinct regulations of release of the two soluble forms. Deletions of the juxtamembrane region of ACE2 reduced LSF release in CHO cell lines, whereas it abolished TACE-induced shedding in EC-2 cells. Analysis of TACE structural domains confirmed that the active site in the catalytic domain is essential for ACE2 shedding but that noncatalytic domains also play additional roles. These results demonstrate selective and specific regulation of constitutive shedding of ACE2 by TACE.

THE RENIN-ANGIOTENSIN SYSTEM (RAS) plays a major role in regulating the cardiovascular, renal, and respiratory systems. Angiotensin-converting enzyme 2 (ACE2), a newly identified component (12, 42), is considered to be a negative regulator of the RAS (10, 20, 29). There is evidence that ACE2 plays an important role in the physiology and pathophysiology of diseases of the heart, kidneys, and lungs (7, 10, 17, 18, 20, 23, 25, 26, 29, 33, 40). Thus understanding regulation of ACE2 could have important therapeutic implications (10, 27).

ACE2 is a type I transmembrane metalloprotease that functions as a carboxypeptidase to hydrolyze substrates such as angiotensin II (12, 42, 43). Structural analysis of ACE2 has revealed the presence of a single catalytic domain that is located in the ectodomain (10, 12, 20, 29, 42). ACE2 has been also identified as a functional receptor for severe acute respiratory syndrome coronavirus (SARS-CoV) (33) and the ectodomain is indispensable to viral attachment through the spike protein (32). Thus the ectodomain is essential for multiple functions of ACE2. Ectodomain shedding of ACE2 results in the proteolytically release of soluble form(s) under various conditions both in vitro (18, 19, 21, 22, 25, 28, 30, 45) and in vivo (14, 25, 31, 38, 40). Previous studies showed that an a disintegrin and metalloprotease (ADAM) family member tumor necrosis factor-α (TNF-α)-converting enzyme (TACE/ADAM17) is involved in this process (19, 30). Similar to ACE2, TACE is a type I transmembrane metalloproteinase, and the biosynthesized initial product consists of multiple domains, including an amino-terminal prodomain, a catalytic domain, a cysteine-rich domain (composed of disintegrin and “epidermal growth factor-like” domains), a transmembrane domain, and a cytoplasmic domain. TACE has a broad somatic distribution and it functions as a “sheddase” for proteolytical cleavage of numerous cell-surface proteins, including receptors and their growth factor cytokine ligands and cell-adhesion molecules (2–4, 6, 11, 13, 16, 36, 39, 41, 44).

There is evidence that TACE-mediated ectodomain shedding influences the biological activities of substrate molecules that have been implicated in regulatory events involved in normal organ function as well as in the pathophysiology of medically important events such as cancer and inflammatory diseases (11, 13, 36, 39, 41). This may be also the case for ACE2. Recently, an increase in circulating soluble ACE2 level was reported in patients with the development of heart failure (14), a finding that may be related to an increase in shedding of this protein. The spike protein of SARS-CoV has been shown to induce TACE-mediated ectodomain shedding of ACE2 (19), and this process has been implicated in the development of respiratory failure. Whereas these reports suggest the pathophysiological significance of ectodomain shedding of ACE2, this process has not been fully characterized. Previous reports indicate that the regulation of TACE-induced shedding of a variety of substrates differ according to the substrate, though substrates may share common mechanisms (3, 13, 39). Moreover, structural elements including the noncatalytic domains of TACE may be involved and their role in shedding is considered to be different dependent on the specific substrates (34, 35, 37).

In the present study we sought to define how TACE specifically regulates ACE2 shedding, focusing on the constitutive process. For this purpose we took advantage of two different pairs of cell lines, in which TACE mutant cells are available. Wild-type Chinese Hamster Ovary (WT-CHO) cells and mutant M2 (M2-CHO) cells were used, based on the fact that in M2-CHO cells the inactive zymogen but not the active mature
form of TACE is detectable, whereas other proteases tested are noted to be correctly processed and intact (1, 5). Mouse embryonic fibroblast cell lines, immortalized EC-4 and EC-2 cells that are derived from wild-type and tace<sup>Znl/A</sup> Znmice, respectively (37), were also used. TACE was reconstituted in EC-2 cells with adenoviral vectors to characterize TACE-mediated shedding of ACE2 and to analyze the functional requirement of the TACE structural domains for ACE2 shedding. Here we show that two major enzymatically active soluble forms are constitutively released from cells expressing ACE2. TACE is mainly responsible for release of one of the soluble forms of ACE2, and there appears to be distinct regulation of release of the two soluble forms. TACE-induced shedding of ACE2 was sensitive to deletions of the juxtamembrane region of ACE2, and both the catalytic and noncatalytic domains of TACE appear to play specific roles in this process.

**MATERIALS AND METHODS**

**Reagents.** Anti-human ACE2 ectodomain monoclonal antibody (MAB933) was purchased from R&D Systems (Minneapolis, MN). Anti-TACE polyclonal antibody (C-15) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-flag M2 monoclonal antibody (MAB933) was purchased from R&D Systems (Minneapolis, MN). Gene Technologies (Rockville, MD). Restriction endonucleases have COOH-terminal Myc-Flag epitope tag, were purchased from OriGene Technologies. A full-length mouse TACE cDNA, cDNA for ADAM10 cDNA, both of which have COOH-terminal Myc-Flag epitope tag, were purchased from Sigma (St. Louis, MO). Anti-actin (20 –33) polyclonal antibody, and synthetic oligonucleotides were purchased from Invitrogen, Carlsbad, CA) was used for cloning of the ACE2 constructs and also for transient transfection described below.

**Table 1. Oligonucleotides used for generation of mutant forms of ACE2 and TACE**

<table>
<thead>
<tr>
<th>Target Constructs</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>ACE2 Δ694-733 (upper)</td>
<td>5′-CTAAACAGCCCGCTTTCTGCA-3′</td>
</tr>
<tr>
<td>ACE2 Δ694-733 (lower)</td>
<td>5′-TATGGGAGGCTATCGGTGGTCGTTG-3′</td>
</tr>
<tr>
<td>ACE2 Δ708-719 (upper)</td>
<td>5′-CGGAAGCGCCTAGGTTTCTGGGATACGCCAACAC-3′</td>
</tr>
<tr>
<td>ACE2 Δ708-719 (lower)</td>
<td>5′-ATTGCTCTATCCCGAAACTCTAGGTTGTCGGGATTG-3′</td>
</tr>
<tr>
<td>ACE2 Δ720-729 (upper)</td>
<td>5′-CAGGCCGATATCTCCATGCTCTGTCGAGACAC-3′</td>
</tr>
<tr>
<td>ACE2 Δ720-729 (lower)</td>
<td>5′-TTGCTCTATCCCGAAACTCTAGGTTGTCGGGATTG-3′</td>
</tr>
<tr>
<td>ACE2 Δ730-739 (upper)</td>
<td>5′-ATGCTCTATCCCGAAACTCTAGGTTGTCGGGATTG-3′</td>
</tr>
<tr>
<td>ACE2 Δ730-739 (lower)</td>
<td>5′-TTGCTCTATCCCGAAACTCTAGGTTGTCGGGATTG-3′</td>
</tr>
<tr>
<td>ACE2 Δcyto (upper)</td>
<td>5′-TATGGGAGGCTATCGGTGGTCGTTG-3′</td>
</tr>
<tr>
<td>ACE2 Δcyto (lower)</td>
<td>5′-CGGAAGCGCCTAGGTTTCTGGGATACGCCAACAC-3′</td>
</tr>
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ACE2, angiotensin-converting enzyme 2; TACE, tumor necrosis factor-α converting enzyme.
ACE2 and TNF-α shedding assays. Forty eight hours after transfection or infection, cells were placed in serum-free medium for 2 h and then washed twice with the medium before starting assays. Cells were then incubated with 1 ml of serum-free medium at 37°C for 6 h unless specified, and the culture media were removed. Cells were washed with ice-cold phosphate-buffered saline and solubilized by incubation at 4°C for 30 min in lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS in phosphate-buffered saline) containing protease inhibitors (10 µg/ml leupeptin, 100 kallikrein inhibitory units/ml aprotinin, and 0.1 mg/ml phenylmethylsulfonyl fluoride). Both culture media and cell lysates were clarified by centrifugation at 4°C for 5 min at 16,000 × g and used for analyses by immunoblotting and/or ACE2 activity assay.

Immunoblotting. Protein concentrations of cell lysates were quantified with a Bio-Rad protein assay (Bio-Rad, Laboratories, Hercules, CA). Culture media were concentrated using Amicon Ultra-4 centrifugal filter devices (Millipore, Billerica, MA). Cell lysates and concentrated media were treated, when necessary, with 1 unit of N-glycanase X (PNGase F, Promega) in 50 mM sodium phosphate buffer (pH 7.5) at 37°C for 3 h, according to the manufacturer’s Solution Digestion Procedures to achieve deglycosylation. An equal amount of protein from cell lysate and an aliquot of concentrated media were separated on 7.5–15% SDS-polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The membrane was immunoblotted using primary antibody described above, followed by horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents (ECL plus, GE Healthcare). Chemiluminescent blots were detected on the Storm 860 scanner (Molecular Dynamics, Sunnyvale, CA) in blue fluorescence mode with a photomultiplier tube voltage of 800 and band intensities were quantified using the attached software (ImageQuant). To assess ACE2 shedding, ratios of the intensities of the soluble forms to the intensity of the cell-associated form from cell lysate on immunoblot were used to normalize for differences in transfection efficiency and for differences in cDNA expression between plates as previously described (37).

ACE2 activity assay. The enzymatic activities of ACE2 in culture media were measured by using the quenched fluorogenic peptide substrate Mca-YVADAPK(Dnp) (R&D systems). Assays were performed in 100 µl of reaction buffer (1 M NaCl, 75 mM Tris, 10 mM ZnCl2, pH 7.4) containing 25 µM substrate at 37°C, in the absence or presence of ACE2 specific inhibitor C16 (1 µM) (9) (a kind gift from Natalie Dales, Millennium Pharmaceuticals, Cambridge, MA) to determine specific enzymatic activity. Fluorescence of hydrolysate from the substrate was monitored for 30 min on a microplate fluorescence reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) with excitation at 320 nm and emission at 450 nm, respectively. The rate of hydrolysis was quantified with a standard curve from definite concentrations of Mca and was used to express enzymatic activity.

Statistics. Data are expressed as means ± SE. Comparisons between three or more groups were performed by one-way analysis of variance, accompanied by post hoc tests as described. Correlation was assessed by r value from the Pearson correlation coefficient. Statistical significance was defined as P < 0.05.
and culture media from WT-CHO and M2-CHO cells, both of which were expressing ACE2. One band with a molecular mass (MM) of ~130 kDa in cell lysates as a cell-associated form (lanes 1 and 3) and two bands with slightly lower MMs of ~110 and ~100 kDa in media as soluble forms (lanes 2 and 4) were detected from WT-CHO and M2-CHO cells. Treatment of samples with PNGase F, an enzyme for the deglycosylation of glycoprotein, revealed that both a cell-associated form and soluble forms are glycosylated, as shown by a shift of the bands to the lower MMs of ~90 kDa from cell lysates (lanes 5 and 7) and of ~80 and ~70 kDa from media (lanes 6 and 8). There was less release of the larger soluble form (LSF) to the smaller soluble form (SSF) noticed in M2-CHO cells when compared with that in WT-CHO cells.

Immunoblots for ACE2 using cell lysates and culture media from WT-CHO cell lines, both of which were overexpressing ACE2, are depicted in Fig. 1B. They showed the band patterns of one cell-associated and two soluble forms similar to those obtained from CHO cell lines in the absence (Fig. 1B, lanes 1–4) or presence (lanes 5–8) of PNGase F treatment. Less release of LSF was noticed in EC-2 cells when compared with that in EC-4 cells. These results suggest that the presence of functional TACE affects ectodomain shedding of ACE2, particularly LSF release in CHO and EC cell lines.

Constitutive release of two soluble forms of ACE2 with MMs similar to those from CHO and EC cell lines was also detected in 293A cells and rat primary cultured cardiomyocytes (Fig. 1C) and in HeLa cells and mouse skin fibroblasts (not depicted), all of which are overexpressing ACE2. These results show that release of the two soluble forms of ACE2 from cells expressing this protein is not limited to only a few cell lines.

Dose- and time-dependent shedding of ACE2 in CHO and EC cell lines. CHO cell lines were transiently transfected with different amounts of ACE2 expression vector, whereas EC cell lines were infected with Ad-ACE2 at different MOIs to examine dose- and time-dependent shedding of ACE2. There was a dose-dependent increase in cell-associated form of ACE2, accompanied by an increased release in soluble forms in both WT-CHO and M2-CHO cells, as detected by immunoblotting (Fig. 2A). Similarly, there was parallel increase in cell-associated form and soluble forms of ACE2 in a MOI-dependent manner in EC-4 and EC-2 cells (Fig. 2B). A time dependency of ACE2 shedding was also demonstrated in both CHO (Fig. 2C) and EC (Fig. 2D) cell lines by collecting media at different time points. At any of the time points lower release of LSF relative to SSF was noted in M2-CHO cells and EC-2 cells compared with that in WT-CHO and EC-4 cells, respectively. Accumulation of soluble forms into media is associated with increasing ACE2 activity (Fig. 2, E and F). Thus these results show dose- and time-dependent shedding of ACE2 and they suggest that TACE is mainly involved in LSF release in CHO and EC cell lines.

Enzymatic activities from the soluble forms of ACE2. To assess enzymatic activity from each soluble form, different patterns of accumulation of the soluble forms into media were obtained from EC-2 cells and WT-CHO cells as shown in Fig. 3A (top). Release of LSF but not SSF from EC-2 cells infected with Ad-ACE2 was obviously increased by TACE reconstitution in these cells by means of concomitant infection with adenovirus encoding TACE with His-Flag (Ad-TACE) according to the MOI indicated (lanes 2–4). Thus reconstitution with TACE increased the shedding to release LSF of ACE2 in EC-2 cells. To assess ACE2 activity from the SSF, a mutant form of ACE2 that has a deletion of amino acids sequence 694-733 in the ectodomain close to the membrane (ACE2 Δ694-733) was generated. In WT-CHO cells transfected with ACE2 Δ694-733 expression vector, SSF but not LSF, was detectable on immunoblots (lane 6), a finding that is in contrast to that seen in WT-CHO cells transfected with WT ACE2 expression vector, which releases two soluble forms (lane 5). Aliquots of culture media were also used for measurements of ACE2 activities (Fig. 3A, bottom). There was increase in ACE2 activity in EC-2 cells infected with Ad-ACE2 that corresponded to increased levels of LSF in immunoblots by TACE reconstitution. There was significant ACE2 activity detected in the medium from cells transfected with ACE2 Δ694-733 expression vector, which released detectable levels of SSF but not LSF on immunoblots (lane 6). An X–Y plot of combined intensities of the LSF and SSF bands on immunoblots and enzymatic activities for ACE2 from culture media demonstrated a strong correlation between combined immunoblot intensities and ACE2 activities ($r^2 = 0.9322$).
These data show that in these cell lines: 1) LSF and SSF have significant and roughly equivalent ACE2 activities, 2) immunoblotting with monoclonal antibody used in the present study would be a useful tool to separately analyze LSF and SSF that are released by ectodomain shedding of ACE2, 3) releases of the LSF and SSF are regulated through distinct mechanisms, and 4) TACE increases ACE2 shedding and is predominantly responsible for release of LSF. These results also suggest that: 1) the two soluble forms shown that were detected are the major soluble forms with enzymatic activities released from cells expressing ACE2, and 2) SSF is likely to be directly produced by cleavage at a distinct site of cell-associated ACE2, rather than by the second cleavage of LSF.

Effects of tandem deletions of the juxtamembrane region of ACE2 on ectodomain shedding in CHO cell lines. A number of the identified sites of cleavage in which TACE is considered to be involved are also located in the juxtamembrane region (3, 16, 41, 44). Thus we focused on this region of ACE2 and examined the effects of tandem deletions directed outward from the membrane on ectodomain shedding in CHO cell lines. Deletions were generated on the amino acid sequence 709-719 (ACE2/H9004709-719), 720-729 (ACE2/H9004720-729) or 730-739 (ACE2/H9004730-739) of ACE2 as shown in Fig. 4A. Both WT-CHO and M2-CHO cells were transiently transfected with expression vector of WT ACE2 or the deletion mutants to examine shedding. As shown in Fig. 4B, in WT-CHO cells that express the ACE2 deletion mutants there was release of LSFs with apparently similar MMs that are lower than that of WT ACE2 as well as release of SSF with MMs similar to that of WT ACE2. These findings indicate that the cleavage site for LSF release does not depend entirely on the presence of a specific amino acid sequence, and they suggest that an interaction between the amino acid sequence and
distance from the membrane influences the cleavage site. Release of LSF was inhibited in ACE2 Δ720-729 and ACE2 Δ730-739, whereas release of SSF was inhibited only in ACE2 Δ730-739 when compared with WT ACE2. As a result, a significant reduction in a total release, which is assessed by a combined intensity of the LSF and SSF on immunoblots, was observed in ACE2 Δ730-739 (Fig. 4B). Reduction of the SSF in ACE2 Δ730-739 suggests the possibility that this deletion induces structural changes in the ACE2 ectodomain, which affect cleavage since the cleavage site for SSF release is expected to be far distant from the deleted regions. A possi-

![Diagram](http://ajpcell.physiology.org/)

Fig. 4. Effects of tandem deletions of the juxtamembrane region of ACE2 on ectodomain shedding in CHO cell lines. A: schematic presentation of tandem deletions of the juxtamembrane region of ACE2. The ACE2 structure is shown above amino acid sequence alignment of the juxtamembrane region of WT ACE2 and the deletion mutants (Δ709-719, Δ720-729, and Δ730-739). The deleted amino acid residues are replaced by dotted lines. Underlines indicate the transmembrane domain (Tm). Cyto, cytoplasmic domain. B: X-Y plot of combined intensities of the LSF and SSF on immunoblots and the corresponding enzymatic activities for ACE2 from culture media. Correlation was assessed by $r^2$ value from the Pearson correlation coefficient.
bility raised by these results is that structural integrity in addition to amino acid sequence is important in determining the susceptibility of ACE2 to shedding in this cell line.

Similar results on effects of the tandem deletions on ACE2 shedding were also obtained in M2-CHO cells with almost complete inhibition of LSF release in ACE2 Δ720-729 and ACE2 Δ730-739 and with significant reduction in a total release in ACE2 Δ730-739 when compared with in WT ACE2 (Fig. 4C).

Effects of tandem deletions of the juxtamembrane region of ACE2 on ectodomain shedding mediated by TACE. To clarify the effects of tandem deletions of the juxtamembrane region of ACE2 on ectodomain shedding mediated by TACE, release of the soluble forms was assessed in EC2 cells infected with WT ACE2 or the deletion mutants in the absence or presence of TACE reconstitution (Fig. 5A). In the absence of reconstitution of TACE, as shown in Fig. 5B on immunoblotting, there was no significant difference in LSF, SSF, or total release between cells infected with Ad-ACE2 and Ad-ACE2 Δ709-719, Ad-ACE2 Δ720-729, or Ad-ACE2 Δ730-739. As shown in Fig. 5C, TACE reconstitution by concomitant infection with Ad-TACE resulted in an increase in release of LSF but not SSF in cells infected with Ad-ACE2 when compared with cells with Ad-ACE2 in the absence of TACE reconstitution (Fig. 5B). In cells infected with Ad-ACE2 Δ709-719, there was an increase in SSF release without significant change in a total release when compared with cells with Ad-ACE2 in the presence of TACE reconstitution (Fig. 5C). In cells with Ad-ACE2 Δ720-729 or Ad-ACE2 Δ730-739, an LSF and total release was inhibited when compared with cells with Ad-ACE2 in the presence of TACE reconstitution (Fig. 5C). Finally, differences in intensities of the bands of the soluble forms on immunoblots obtained in the absence and presence of TACE reconstitution were calculated to assess the net effects of the reconstitution on ACE2 shedding. As shown in Fig. 5D, TACE reconstitution was found to predominantly stimulate LSF release with increase in a total release from cells infected with Ad-ACE2. In cells with Ad-ACE2 Δ709-719, TACE reconstitution increased total release of the soluble forms to the same extent, accompanied by less LSF and more SSF release when compared with Ad-ACE2. In cells with Ad-ACE2 Δ720-729 or Ad-ACE2 Δ730-739, TACE reconsti-

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**Fig. 5.** Effects of tandem deletions of the juxtamembrane region of ACE2 on ectodomain shedding in EC2 cells in the absence or presence of tumor necrosis factor-α-converting enzyme (TACE) reconstitution. EC2 cells were infected with adenovirus encoding WT ACE2 (Ad-ACE2) or the deletion mutant (Δ709-719, Δ720-729, or Δ730-739) at MOI of 10, in the absence or presence of reconstitution of TACE by concomitant infection with empty adenovirus or adenovirus encoding TACE, respectively, at MOI of 20. ACE2 shedding assays were performed for 6 h. A: TACE expression in EC2 cells in the absence (TACE (-)) or presence (TACE (+)) of TACE reconstitution. Immunoblots for TACE as well as actin using cell lysates from EC2 cells infected with indicated adenoviruses were depicted. B and C: ectodomain shedding of WT ACE2 and the deletion mutants in EC2 cells in the absence (B) or presence (C) of reconstitution of TACE. Representative immunoblots for the ectodomain of ACE2 using cell lysates (C) and culture media (M) (without PNGase F treatment) are depicted above the composite data on relative intensities of the bands. The relative intensities of the bands for the LSF and SSF soluble forms from culture medium are expressed as ratios to the intensity of the cell-associated form from cell lysate. A combined intensity of LSF and SSF (combined) from cells infected with Ad-ACE2 in the absence of TACE reconstitution was defined as 1.00. D: effects of TACE reconstitution on ectodomain shedding of WT ACE2 and the deletion mutants in EC2 cells. Differences in LSF, SSF, and combined intensities were obtained by subtracting the values of intensities in the absence of reconstitution from those in the presence of reconstitution to assess the net effects of reconstitution. Data are means ± SE (n = 5). *P < 0.05, †P < 0.01 vs. the corresponding soluble form or combined intensity in cells infected with Ad-ACE2 (Dunnett’s post hoc test).
tution did not have significant effects on LSF or SSF release, showing that the deletions almost completely abolish a LSF and total release induced by TACE.

To further investigate the effects of TACE-induced shedding in EC2 cells, tandem Ala mutants carrying point mutations of the amino acid sequence 730-739 of ACE2 were constructed. However, none of these point mutations had any significant inhibitory effects on TACE-induced shedding (data not shown). These results suggest the importance of structural integrity rather than the specific amino acid sequence of the juxtamembrane region of ACE2 in determining the susceptibility to shedding also in EC2 cells.

Analysis of the functional domains of TACE for ectodomain shedding of ACE2. Whereas a number of proteins have been identified as substrates for TACE-mediated shedding, previous reports suggest that the structural elements of TACE involved in each shedding event may differ depending on the particular substrate and/or stimulation (34, 35, 37). Thus the functional requirement of the TACE structural domains for constitutive ACE2 shedding was investigated using truncation (TACE ΔCyto), mutation (TACE E406A), and chimeric constructs of TACE (TACE Pro-Cat/ADAM10 and TACE Cyto/ADAM10) as well as ADAM10, which shares the same domain structure and has a high amino acid sequence homology with TACE, as described in Fig. 6A. ACE2 shedding was evaluated by immunoblotting using media from EC2 cells coinfected with Ad-ACE2 and adenoviruses encoding ADAM constructs. As shown in Fig. 6B, expression of ADAM constructs was confirmed by immunoblot for Flag tag. Infection with Ad-TACE increased release of LSF but not SSF with increased total release of the soluble forms of ACE2 (lane 3) when compared with empty virus (Ad-CTL) infection (lane 2). Ad-TACE ΔCyto infection also increased LSF and total release similar to

Fig. 6. Analysis of the functional domains of TACE for ectodomain shedding of ACE2 and TNF-α. A: schematic presentation of the domain structure of a disintegrin and metalloprotease (ADAM) constructs. Constructs were generated as described in MATERIALS AND METHODS and tagged at the COOH-terminus. Domains from TACE and ADAM10 are indicated by solid bar and open bar, respectively. A point mutation is indicated by hatched area. Pro, prodomain; Cat, catalytic domain; Cys-rich, cystine-rich domain; Tm, transmembrane domain; Cyto, cytoplasmic domain. B: ectodomain shedding of ACE2 in EC-2 cells coinfected with Ad-CTL (CTL) or Ad-ACE2 (at MOI of 20) and with Ad-CTL or adenovirus encoding ADAM construct, Ad-TACE (TACE), Ad-TACE ΔCyto (TACE ΔCyto), Ad-TACE E406A (TACE E406A), Ad-TACE Pro-Cat/ADAM10, Ad-TACE Cyto/ADAM10 or Ad-TACE Cyto/ADAM10 (TACE Cys/ADAM10) (at MOI of 10). ACE2 shedding assays were performed for 6 h. Representative immunoblots for the ectodomain ACE2 using cell lysates (cell) and culture media (medium) and for Flag epitope and actin using cell lysates from EC-2 cells are depicted above the composite data on relative intensities of the bands for the soluble forms. The relative intensities of the bands of the LSF and SSF soluble forms from culture medium is expressed as ratio to the intensity of the cell-associated form. A intensity of the soluble form from cells coinfected with Ad-TNF-α and Ad-CTL was defined as 1.00. Data are means ± SE (n = 4). *P < 0.001 vs. the corresponding soluble form in cells with Ad-TNF-α plus Ad-CTL (Bonferroni’s post hoc test).
Ad-TACE infection, whereas SSF release was considerably less than that with Ad-TACE infection (lane 4). Ad-TACE Pro-Cat/ADAM10 infection increased a LSF and total release compared with Ad-CTL infection, though not so much as with Ad-TACE infection (lane 6). Infection with Ad-TACE E406A (lane 5), Ad-ADAM10 (lane 7) or Ad-TACE Cyto/ADAM10 (lane 8) did not have significant effects on a LSF, SSF, or total release. These results showed that the active site in the catalytic domain of TACE is essential for LSF release. They also suggest that the cysteine-rich and/or transmembrane domains and the cytoplasmic domain play additional but significant roles in regulating ACE2 shedding.

To examine specificity of the functional requirement of each TACE domain for ACE2 shedding, analysis of TNF-α shedding using the ADAM constructs was also performed in EC2 cells. As shown in Fig. 6C, a single soluble form was detectable from culture media in EC2 cells infected with adenovirus encoding TNF-α with COOH-terminal Myc-Flag tag (Ad-TNF-α) by immunoblotting for Myc (lanes 2–8). Concomitant infection with Ad-TACE (lane 3), Ad-TACE ΔCyto (lane 4), or Ad-TACE Pro-Cat/ADAM10 (lane 6), as is the case with ACE2 shedding, increased TNF-α release into the media compared with Ad-CTL infection (lane 2). However, there was no difference in release of the soluble form of TNF-α between cells coinfected with Ad-TACE, Ad-TACE ΔCyto or Ad-TACE Pro-Cat/ADAM10, in contrast to the case with ACE2. These results suggest a particular role of the cysteine-rich and/or transmembrane domains of TACE in ACE2 shedding for LSF release.

To further investigate the role of the cytoplasmic domain of TACE, a truncated form of ACE2 lacking in the cytoplasmic domain (ACE2 ΔCyto) was tested for shedding in EC2 cells in the absence or presence of TACE or TACE ΔCyto reconstitution. As shown in Fig. 7, cells infected with adenovirus encoding ACE2 ΔCyto (Ad-ACE2 ΔCyto) (Fig. 7A, lane 5) compared with cells with Ad-ACE2 (lane 2) had increased LSF, SSF, and total release, which is consistent with previous reports (25, 28). In cells with Ad-ACE2 ΔCyto, coinfection with Ad-TACE (lane 6) or Ad-TACE ΔCyto (lane 7) further increased LSF and total release, whereas SSF release was less in cells with Ad-TACE ΔCyto than that with Ad-TACE, as is observed in cells with Ad-ACE2 (Fig. 7A, lanes 3 and 4, and Fig. 6B, lanes 3 and 4). These results suggest that the cytoplasmic domain of TACE may have a potential role in indirectly regulating constitutive SSF release, independent of existence of the cytoplasmic domain of ACE2. However, since a chimera TACE Cyto/ADAM10 did not have any effects on SSF release (Fig. 6B, lane 8), the possibility that the other domains of TACE may support this role of the cytoplasmic domain is suggested.

Thus, taken together, the results from analysis of the TACE domains indicate that both the catalytic and noncatalytic domains of TACE play specific role in ectodomain shedding of ACE2.

DISCUSSION

The major findings at the present study are that: 1) there are two distinct major soluble forms of ACE2 released from CHO and EC cell lines overexpressing this ectoenzyme, both of which are detected by immunoblotting and which have similar enzymatic activities; 2) TACE is mainly responsible for release of the LSF of ACE2; 3) deletions of the juxtamembrane region of ACE2 attenuates TACE-induced shedding; and 4) the active site in the catalytic domain of TACE is essential for ACE2 shedding, whereas noncatalytic domains play additional and specific roles. In our study two major bands were constantly detected by immunoblotting using monoclonal antibodies against the ectodomain of ACE2 from serum-free media of both unstimulated CHO and EC cell lines and other cell types overexpressing this protein. Our results are consistent with a recent report that ACE2 is shed to release two soluble forms in pulmonary epithelial cells in vivo in humans (25). However, previous studies have not separately analyzed the two soluble released forms.

**Fig. 7.** Effects of a deletion of the cytoplasmic region of TACE on ectodomain shedding in EC2 cells in the absence or presence of the cytoplasmic region of ACE2. EC-2 cells were coinfected with Ad-CTL (CTL) or adenovirus encoding ACE2 construct, Ad-ACE2 (WT) or Ad-ACE2 ΔCyto (ΔCyto) (at MOI of 20), and with Ad-CTL or adenovirus encoding TACE construct, Ad-TACE (TACE) or Ad-TACE ΔCyto (TACE ΔCyto) (at MOI of 10). ACE2 shedding assays were performed for 6 h. A: representative immunoblots for the ectodomain ACE2 using cell lysates (Cell) and culture media (Medium) from EC2 cells. B: the composite data on relative intensities of the bands for the soluble forms from cells with Ad-ACE2 ΔCyto. The relative intensities of the bands of the LSF and SSF soluble forms from culture medium are expressed as ratios to the intensity of the cell associated-form from cell lysate. A combined intensity of LSF and SSF soluble forms from culture medium are expressed as ratios to the intensity of the cell associated-form.
The TACE mutant CHO-M2 and EC-2 cells are particularly useful to probe the mechanisms controlling ectodomain shedding by this sheddase. In these two different cell lines, reduced LSF release of ACE2 was found compared with that in WT-CHO and EC-4 cells, respectively, which contain functional TACE. These results suggest that TACE at least in part independently cleaves ACE2 to release of LSF. However, since some release of LSF and SSF was seen in both TACE-deficient M2-CHO and EC-2 cells, it appears that TACE is not the only sheddase responsible for ACE2 shedding. The findings in the present study that deletion of amino acid sequence 694-733 (Fig. 3A) or 720-729 (Fig. 4, B and C) of ACE2 resulted in inhibition of release of LSF but not SSF in CHO cell lines and that TACE reconstitution in EC2 cells stimulated LSF but not SSF release show distinct regulations of release of the two soluble forms. Thus TACE is considered to be a primary sheddase responsible for release of the LSF of ACE2, whereas it is speculated that other sheddase(s) are involved in release of SSF and to a lesser degree of the LSF.

TACE cleaves a number of membrane proteins including both type I and type II topology. The known sites of cleavage in which TACE is considered to be involved are generally located at the juxtamembrane region (3, 16, 41, 44). Mutational analysis of the cleavage sites of substrates have revealed relaxed sequence specificities surrounding the cleavage sites, though some preference sequences are noted (2, 3, 16, 39, 41, 44). Our results showing that deletions of 9−10 amino acids, but not specific point mutations, of the juxtamembrane region of ACE2 have inhibitory effects on ectodomain shedding in EC2 cells in the presence of TACE reconstitution (Fig. 5, B and C) is consistent with previous reports suggesting that the structure of the juxtamembrane stalk region, rather than the presence or absence of a specific amino acid sequence, determines the susceptibility of a transmembrane protein to shedding (16, 41, 44). However, potential effects of change in structural integrity on ectodomain shedding appear to differ depending on cell types and their sheddase expression, since there is difference in effects of deletions of the juxtamembrane region of ACE2 between CHO cell lines and EC2 cells and also between the results in the present study and in a recent report (25).

Our results confirmed that the amino acid sequence at the active site in the catalytic domain of TACE is indispensable to shedding of ACE2, as is the other substrates of TACE (15, 34). However, they also indicate that noncatalytic domains, including the pro-, cysteine-rich, transmembrane, and cytoplasmic domain, also play role in full and specific TACE activity involved in cleavage of ACE2. Since the maturation process of TACE by removal of the prodomain is impaired in CHO-M2 cells (5, 37), impaired LSF release in this cell line compared with in WT-CHO cells indicates that this process is a first key step required for TACE to cleave ACE2. mRNA compatible with TACE Δcyto has been detected and it is considered to be generated by alternative splicing (6, 37). In the present study there was no difference in a total release of the soluble forms of ACE2 as assessed by a combined intensity of the LSF and SSF on immunoblots between reconstitution of wild-type (full-length) TACE and TACE Δcyto in EC2 cells. The results are consistent with previous reports showing that deletion of the cytoplasmic domain of TACE has no detectable effects on a total release of the soluble forms of substrates, including TNF-α, the p75 TNF-α receptor (p75 TNFR), the type II interleukin-1 receptor (IL-1R-II) (37), and transforming growth factor-α (TGF-α) (15). We found, however, that there was a reduction in SSF release with TACE Δcyto reconstitution compared with wild-type TACE, whereas TACE Cyto/ADAM10 did not have any effects on SSF release. The effect of a deletion of the cytoplasmic domain of TACE on SSF remained regardless of the presence of the cytoplasmic region of ACE2. These results suggest the possibility that the cytoplasmic domain in connection with the other domains of TACE interacts with other sheddase(s) involved in constitutive release of SSF and affects their sheddase activities (13, 39) to indirectly regulate SSF release in EC2 cells. Nevertheless, we cannot exclude the possibility that interactions between ACE2 and TACE were induced through the cytoplasmic domains in specific situations such as viral attachment to ACE2 as a previous report suggested (19). A chimera TACE Pro-Cat/ADAM10 increased a LSF and total release of ACE2 while the release was less than that induced by TACE in EC2 cells. Reddy et al. (37) showed that, similar to what we found for ACE2, the cysteine-rich domain is required for full TACE activity to cleave IL-1R-II but not for TNF-α or p75 TNFR cleavage. Previous reports showed that both the cysteine-rich and transmembrane domain of TACE could have a functional role such as substrate recognition in shedding depending on substrates (34, 35, 37). The difference in effects on ACE2 release between by TACE Pro-Cat/ADAM10 and by TACE in the present study suggests that the particular amino acid sequence of this region of TACE could encode important information specific for ACE2 shedding, on the assumption that the domain structure is conserved even in the chimeric construct (34).

Based on the results in the present study, it is speculated that there might be a predominant increase in release of LSF of ACE2 under certain conditions where TACE activities increased in tissues expressing ACE2. We need to further examine TACE-mediated ACE2 shedding under stimulated condition and involvement of other sheddase(s) responsible for shedding of this protein. Since ACE2 shedding could result in a shift of a balance between angiotensin-converting enzyme and ACE2 activities and in alteration in local concentrations of angiotensin peptides in a microenvironment around cells, shedding of this protein could affects pathogenesis in tissues or organs, as is the case with other substrate of TACE (11, 13, 36, 39, 41), though the long-term effects of this event should be evaluated. It is noted in the present study that the LSF and SSF have equivalent enzymatic activities. We cannot, however, exclude the possibility that there is a subtle difference in enzymatic activity between the two soluble forms. Further characterization of each soluble form of ACE2 in the view of binding capacity to viral protein and other potential biological activities would be interesting and could provide another significance of studies on ACE2 shedding. In addition, delivery of modified forms of ACE2 for ectodomain shedding or certain soluble forms that have binding capacity to pathogenic viruses might have therapeutic implications (10, 21, 27).

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