The collectrin-like part of the SARS-CoV-1 and -2 receptor ACE2 is shed by the metalloproteinases ADAM10 and ADAM17

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Abstract
The transmembrane protease angiotensin converting enzyme 2 (ACE2) is a protective regulator within the renin angiotensin system and additionally represents the cellular receptor for SARS-CoV. The release of soluble ACE2 (sACE2) from the cell surface is hence believed to be a crucial part of its (patho)physiological functions, as both, ACE2 protease activity and SARS-CoV binding ability, are transferred from the cell membrane to body fluids. Yet, the molecular sources of sACE2 are still not completely investigated. In this study, we show different sources and prerequisites for the release of sACE2 from the cell membrane. By using inhibitors as well as CRISPR/Cas9-derived cells, we demonstrated that, in addition to the metalloprotease ADAM17, also ADAM10 is an important novel shedding protease of ACE2. Moreover, we observed that ACE2 can also be released in extracellular vesicles. The degree of either ADAM10- or ADAM17-mediated ACE2 shedding is dependent on stimulatory conditions and on the expression level of the pro-inflammatory ADAM17 regulator iRhom2. Finally, by using structural analysis and in vitro verification, we determined for the first time that the susceptibility to ADAM10- and ADAM17-mediated shedding is mediated by the collectrin-like part of ACE2. Overall, our findings give novel insights into sACE2 release by several independent molecular mechanisms.

KEYWORDS
ADAM10, ADAM17, ectodomain shedding, SARS-CoV receptor, soluble ACE2

Abbreviations: ACE2, angiotensin converting enzyme 2; ADAM, a disintegrin and metalloproteinases; Ang, angiotensin; BTC, betacellulin; CLD, collectrin-like domain; CLP, collectrin-like part; CoV, coronavirus; MD, metalloprotease domain; RAS, renin angiotensin system; sACE2, soluble ACE2; SARS, severe acute respiratory syndrome; TGFs, transforming growth factor.

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1 INTRODUCTION

The type 1 transmembrane metalloprotease angiotensin converting enzyme 2 (ACE2) is involved in many (patho)physiological processes. ACE2 represents the counterbalance of ACE within the renin angiotensin system (RAS). Here, the peptide hormone angiotensin II generated by ACE is further converted by ACE2 into angiotensin1–7.1,2

Thereby ACE2 promotes vasodilation, anti-fibrotic, anti-proliferative and anti-inflammatory effects. Several mouse models with ACE2-deficiencies also show the numerous protective functions of ACE2 such as the prevention of Angiotensin II-mediated heart failure, vascular dysfunctions and renal fibrosis.3

Importantly, ACE2 represents the cell-tethered receptor for the spike protein of SARS coronavirus (CoV)-1 and -2 and is hence crucial for the viral cell entry.4,5

Soluble ACE2 (sACE2) can be found circulating in humans and mice and one of the main sources seems to be kidney tissue.6–11 Yet, the origin and functional consequences of sACE2 are not fully understood. The level of sACE2 is elevated in pathologies such as chronic kidney disease and cardiovascular disease.6,9,12 Membrane proteins can undergo ectodomain shedding, a process in which its ectodomain can be cleaved by proteases called sheddases. Previously, the metalloprotease ADAM17 (a disintegrin and metalloproteinases 17) has been indicated to act as an ACE2 sheddase.7,13–15 In contrast, ACE2 cleavage by the serine protease TMPRSS2 does not lead to sACE2.14 Overall, many questions remain to be answered regarding the molecular basis of sACE2 generation, including the structural requirements for the release, the position of the cleavage sites and possible additional sources of sACE2.

ACE2 is a chimeric fusion gene consisting of the C-terminal catalytically active metalloprotease domain (MD) of the ACE gene and almost all parts of the collectrin gene (TMEM27). While the positions of putative cleavage sites are still unclear, the MD was described to be a necessary structural determinant for shedding, since the collectrin-like part (CLP) alone was previously described as shedding resistant13,15

Importantly, the release of sACE2 seems to be crucial for its biology, as sACE2 provides its activity in tissues with low ACE2 expression.9 sACE2 is also discussed in terms of modulating the susceptibility of cells to infection by CoV16,17 (Figure 1A). It is still debated whether sACE2 promotes or prevents CoV infection and the subsequent manifestation of the severe acute respiratory syndrome (SARS). Strikingly, soluble ACE2 was recently described to promote SARS-CoV infection of cells with low ACE2 expression.17 Hence, elucidating the molecular mechanism of ACE2 release is of critical importance.

ADAM17 and its close relative ADAM10 are prominent sheddases, since they are involved in various (patho)physiological processes such as development, regeneration and immunity.18–21 Here, both proteases are involved in the proteolytic release of different cytokines or their receptors such as interleukin-6 receptor (IL6R), tumor necrosis factor (TNFα), CXCL16 and CX3CL1, EGFR ligands such as betacellulin (BTC), transforming growth factor (TGFα) and amphiregulin as well as adhesion molecules such as E-cadherin and JAM-A.22–27

ADAM10 and ADAM17 are synthesised as proforms into the ER with an inhibitory prodomain. Maturation of these proforms takes place in the Golgi by furin-like pro-protein convertases. Mature ADAM10 and ADAM17 can then be transported to the cell surface, where the shedding process takes place. Cell-surface shedding activity of both proteases is dependent on and regulated by different cellular signals, protein interactions and the membrane compositions, which leads to structural changes within the proteases.28–33 ADAM10 activity seems to be primarily dependent on Ca2+ influx and calmodulin inhibition.34–36 Hence, ADAM10 activity can be increased by the use of the ionophore ionomycin. ADAM17 activity seems to be heavily dependent on PKC activity.28,34–36 Thus, phorbol esters such as PMA (phorbol 12-myristate 13-acetate), which non-specifically activate PKCs, increase mainly ADAM17-mediated shedding. While ADAM10 is considered to be mainly constitutively active, suggesting cell processes that stimulate ADAM10 constitutively, the activity and cell surface expression of ADAM17 are often stimulated by various stimuli such as inflammatory signals.39,20,32,37 Under inflammatory conditions the main driver of ADAM17 activity seems to be its regulator the pseudoprotease iRhom2 that promotes trafficking of the protease from the ER to the Golgi and also stabilises ADAM17 at the cell surface.38–40

So far, ADAM17 is believed to be the main ACE2 sheddase, while the involvement of ADAM10 in ACE2 shedding is unclear. Furthermore, ACE2 shedding is not yet understood in terms of the structural requirements of ACE2 and its regulation by iRhom2.

We found that ACE2 is very lowly expressed in most human tissues, whereas it is highly expressed in the kidney, where ADAM10 is also highly expressed. In this study we provide multiple lines of evidence that ADAM10 is a major ACE2 sheddase leading to the release of active sACE2. We also observed that a significant degree of ACE2 is released in an ADAM10- and ADAM17-independent manner via the release of extracellular vesicles. Furthermore, we found that increased iRhom2 expression facilitates a switch from ADAM10- to ADAM17-mediated shedding of ACE2. Finally, with structural and in silico analysis, we determined the borders between the different structural
FIGURE 1  ACE2 is shed by ADAM17 and ADAM10. (A) Overview of ACE2 biology. For quantitative analysis of release ACE2 was fused with AP (AP-ACE2). (B–F) HEK293 cells (B and C) and HEK293 cells deficient for ADAM10 (ADAM10 KO), ADAM17 (ADAM17 KO) or both (A17/A10 KO) (D–F), transiently expressing AP-ACE2 were used to analyse ACE2 release via AP assay. Cells were treated as labelled: 100 nM PMA; 40 µM TAPI1; 10 µM GI; 25 µM Ma, and incubated for 24 h. n ≥ 3
subunits of ACE2. In contrast to previous reports, here we were able to prove for the first time that the collectrin-like part (CLP) of ACE2 alone is susceptible to ADAM17- and ADAM10-mediated shedding, using these newly defined domain boundaries.

2 | MATERIALS AND METHODS

2.1 | Cell culture

HEK293 cells (from ATCC), TC7 cells (from ATCC) and HEK293 cells with CRISPR/Cas9 introduced deficiency for ADAM10, ADAM17 or both33 were cultured in a humidified incubator at 37°C with 5% CO₂ in DMEM5%. DMEM5% contains DMEM high-glucose (Merck, Darmstadt, Germany) supplemented with 5% foetal calf serum (PanBiotechm, Aidenbach, Germany), 100 mg/L streptomycin (Merck, Darmstadt, Germany) and 60 mg/l penicillin (Merck, Darmstadt, Germany). To produce stably expressing cells the pMOWS-vector system41 combined with the Phoenix amphi cells, a virus-producing cell line (HEK293 cell derivate; ATCC CRL-3213, ATCC, Manassas, Virginia, USA) for cell transductions, was used.42

2.2 | Structure and sequence analyses

Sequence alignments and secondary structure predictions were performed with the webtool Clustal Omega,43 PRALINE44 and Jpred4.45 Structural analysis was performed with UCSF ChimeraX46 using the ACE2 structure pdb: 6m1d.47

2.3 | Cloning

The constructs 4xmycAP-ACE2-pMOWS_Zeo, AP-ACE2-pMOWSCMV_Zeo, AP-CLP-pMOWSCMV_Zeo and 4xmycAP-CLP-pMOWS_Zeo were produced by fusing the human placenta alkaline phosphatase with or without 4xmyc-tag to the N-terminus of the full length human ACE2 without the signal peptide (from pCG1-hACE25 kindly provided by Prof. Pöhlm, German Primate Centre, Göttingen, Germany) or the collectrin-like part of ACE2 (CLP) starting with K619, respectively, by using overlapping PCR.48 Wt ACE2 and all ACE2 constructs were cloned into the pMOWS or pMOWSCMV (additional CMV promoter) backbone with zeocin resistance41,42 via the NEBuilder HiFi DNA assembly master mix (E2621L, NEB, Ipswich, Massachusetts, USA) according to the manufacturer's manual. pcDNA3.1 expression plasmids encoding HA-tagged mouse RHBDL1, RHBDL2, RHBDL3, and RHBDL4 were used by us previously49 and originally provided by Dr Matthew Freeman (Sir William Dunn School of Pathology, Oxford, UK).50

2.4 | AP assay

Shedding of AP-ACE2, AP-CLP, 4xmycAP-ACE2, 4xmycAP-CLP, AP-TGFα and AP-BTC was measured by an alkaline phosphatase (AP)–based assay. Here, HEK293 cells, TC7 cells or HEK293 cells deficient for ADAM17, ADAM10 or both33 either transiently or stably expressing the substrate of interest were used. Production of stably expressing cells was described above. Transient transfection was performed via the use of Lipofectamine 3000 (L3000015, Thermo, Waltham, Massachusetts, USA) according to the manufacturer's manual. Inhibition and stimulation of the cells was performed in 6-well plates at a confluency of around 80%. Cells were inhibited as described in detail in the figure legends. Used inhibitors: 40 µM or 10 µM TAPI1 (S7434, Selleck Chemicals, Houston, Texas, USA), 10 µM GI254023X (synthesised, Ludwig et al.51), 25 µM marimastat (M2699, Merck, Darmstadt, Germany). Used stimulators: 100 nM PMA (Cay10008014, Cayman Chemical, Ann Arbor, Michigan, USA), 1 µM ionomycin (i0634, Merck, Darmstadt, Germany) in Gibco™ OptiMEM (11058–021, Thermo, Waltham, Massachusetts, USA) for 24 h or in serum-free medium (DMEM) for 2 h at 37°C. The shedding activity was assessed by measuring the AP activity in the supernatant and in cell lysates. Used lysis buffer: 50 mM Tris; 1% Triton X-100; 150 mM NaCl; 2 mM EDTA; 10 mM 1,10-phenanthroline (37620, Thermo, Waltham, Massachusetts, USA); 1 tablet Complete protease inhibitor (1169748001, Merck, Darmstadt, Germany); pH7.5. p-Nitrophenyl phosphate (PNPP) solution (37620, Thermo, Waltham, Massachusetts, USA) was added to continuously measure the AP activity at 405 nm with the FLUOstar Optima (BMG LABTECH, Ortenberg, Germany). To assess the AP activity the slope (change of absorption at 405 nm per ms) was calculated. The relative shedding activity was calculated as PNPP substrate turnover (AP activity) in the supernatant in relation to the total turnover in supernatant plus cell lysate (total amount of available substrate).

2.5 | ACE2 activity assay

HEK293 cells and HEK293 cells stably expressing murine iRhom242 were transiently transfected with wtACE2_pMOWSCMV as described above. At a confluency of 90% in a 24-well plate cells were treated with...
stimulators and inhibitors as described above for 2 h at 37°C. Supernatant was harvested and cell were lysed in ACE2 activity assay lysis buffer (50 mM Tris; 1% Triton X-100; 137 mM NaCl; 2 mM EDTA; 5 mM ZnCl₂; 1 tablet complete protease inhibitor; pH7.5). Assay solution contains 45 µl reaction buffer (50 mM Tris; 300 mM NaCl; 0.1 mM ZnCl₂; 0.01% Triton X-100; adjust pH6.5) and 5 µl 0.1 mM fluorogenic ACE2 peptide substrate (Mca-YVADPK(Dnp)-OH; ES007, R&D systems, Minneapolis, Minnesota, USA) in DMSO. 50 µl supernatant or 5 µl cell lysate were mixed with 50 µl assay solution and increase in fluorescence was continuously measured (ex: 320 nm; em: 405 nm) with the FLUOstar Optima. The relative ACE2 activity was calculated as substrate turnover (slope of the fluorescence increase) in the supernatant in relation to the total turnover in supernatant plus cell lysate (total amount of available substrate).

2.6 Extraction of extracellular vesicles

The procedure of extraction of extracellular vesicles was performed as described before: At a confluency of around 80% cells of interest (2 × 15 cm dish) were cultured in serum-free medium for 24 h. Supernatant was collected and centrifuged for 10 min at 300 g at 4°C, followed by centrifugation for 20 min at 2000 g and 30 min at 10 000 g at 4°C (ultracentrifuge L7-65, Beckman, rotor type 70 Ti, Beckman Coulter Pasadena, California, USA). Afterwards, the supernatant was filtered through a 0.22 µm filter and then centrifuged for 75 min at 100 000 g at 4°C. The sediment was re-suspended in 15 ml ice-cold PBS (17-512F/12 882104, Lonza, Basel, Switzerland). The suspension was centrifuged for 75 min at 100 000 g at 4°C. Supernatant was removed and the pellet containing extracellular vesicles was dried. The extracellular vesicles were resuspended in 40 µl lysis buffer (20 mM Tris, 150 mM NaCl, 1 tablet complete protease inhibitor, 1% Triton X-100, 1 mM PMSF, 10 mM 1,10-phenanthroline, 1 mM EDTA, 1 mM Na₂VO₄, 10 µM GI254023X). This solution was prepared for analysis via western blot by adding 10 µl reducing loading buffer (3% (w/v) SDS, 16% glycerol, 8% 2-mercaptopethanol, 0.01% (w/v) bromphenol blue, 0.1 M Tris HCl, pH 6.8) and heating it at 65°C for 20 min. As input control, cells were lysed in lysis buffer (50 mM Tris; 1% Triton X-100; 150 mM NaCl; 2 mM EDTA; 10 mM 1,10-phenanthroline; 1 tablet complete protease inhibitor; pH7.5) for 40 min at 4°C and then centrifuged for 20 min at 16 000 g 4°C. For western blotting 40 µl of lysate were mixed with 10 µl reducing loading buffer and heated at 65°C for 20 min.

2.7 RHDBL2-mediated shedding

Proteolysis of ACE2 by rhomboid proteases in HEK293 cells was analysed as described previously. In brief, 1.8 × 10⁶ HEK293 cells per cell culture dish were seeded in 10 cm cell culture dishes 24 h before transfection. Cells were transiently transfected with the respective expression plasmids using TurboFect Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. 48 h after transfection, the medium was replaced with 5 ml serum-free medium and cells and supernatant were collected 4 h later. After sterile filtration, proteins in the supernatant were precipitated using 20% trichloroacetic acid. After centrifugation for 20 min at 18 000 g, pellets were washed with acetone, centrifuged again for 20 min at 18 000 g and dried afterwards. Subsequently, the pellets were boiled in Laemmli buffer for 5 min at 96°C in preparation for western blotting. The cells were washed with PBS (140 mM NaCl, 2.7 mM KCl, 80 mM Na2HPO4×2 H2O, 1.5 mM KH2PO4) and lysed in 400 µl lysis buffer consisting of 10 mM Tris-HCl pH 7.5, 140 mM NaCl, 5 mM EDTA pH 8.0, 1% Triton-X-100, 1% protease inhibitor cocktail set III, EDTA-free (Calbiochem/Merck Millipore, Burlington, MA, USA) and 1% phosphatase inhibitor cocktail PhosSTOP (Roche, Basel, Switzerland). Cell lysates were also boiled in Laemmli buffer for 5 min at 96°C.

2.8 Western blotting

After SDS-PAGE, proteins were transferred onto a PVDF membrane with pore size 0.45 µm (IPFL00010, Immobilon®-FL, Millipore, Burlington, Massachusetts, USA) or onto a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA) by western blotting. The membrane was blocked with 5% (w/v) non-fat dry milk in TBS (50 mM Tris, 150 mM NaCl, pH7.4) with 0.1% Tween-20 (TBST) for 20–30 min at room temperature. This was followed by three washing steps with TBST. Thereafter, primary antibody was applied to the membrane: either for 2 h at room temperature or overnight at 4°C. The membrane was washed with TBST three times and then incubated with secondary antibody for 1 h at room temperature. The membrane was washed with TBST once and with TBS twice. For fluorescence- or chemiluminescence-based detection of proteins, the ChemiDoc™ MP Imaging System (Bio-Rad Laboratories Inc., Hercules, California, USA) was used. Quantification of bands was determined with Image Studio Lite (LI-COR Biosciences, Lincoln, Nebraska, USA). In case of using chemiluminescence for detection, an ECL-solution (ratio of solutions 1:1, Amersham ECL Prime Western-Blot-System, RPN2232,
Thermo, Waltham, Massachusetts, USA) was used. The following primary antibodies with the corresponding dilution were applied to the membrane: α-GAPDH (1:2000 in PBS with 0.1% Tween-20 and 5% non-fat milk powder, MA5-15738, Thermo, Waltham, Massachusetts, USA), α-HSP70 (1:1000 in TBST and 5% non-fat milk powder, EXOAB-HSP70A-1, SBI System Biosciences, Palo Alto, California, USA), α-Myc (1:5000 in TBST and 1% BSA, ab32, Abcam, Cambridge, United Kingdom), HRP-conjugated α-Myc (1:1000 in TBST and 1% BSA, ab19312, Abcam, Cambridge, United Kingdom), α-transferrin receptor (1 µg/ml in TBST and 1% BSA, ab84036, Abcam, Cambridge, United Kingdom), α-ADAM10 (1:1000 in TBST and 1% BSA, ab39162, Abcam, Cambridge, United Kingdom), α-ADAM17 (1:1000 in TBST and 1% BSA, ab39162, Abcam, Cambridge, United Kingdom), α-HA (1:2000 in TBST and 1% BSA, 901502, Biolegend, San Diego, California, USA). The following secondary antibodies with the indicated dilutions in PBS with 0.2% BSA added were used: αADAM17 (1:100, MAB 9301, R&D Systems, Minneapolis, Minnesota, USA), αHA (1:500, 901502, Biolegend, San Diego, California, USA). The following secondary antibodies with the indicated dilutions were used: allophycocyanin-conjugated mouse (1:200, 115-135-164, Jackson ImmunoResearch, West Grove, Pennsylvania, USA).

2.10 | Gene expression analysis

For mRNA expression analysis in different human tissues of healthy human samples the transcriptome data from different public repositories, which were generated with Affymetrix Human Genome U133Plus 2.0 Arrays, were studied using the Genevestigator suite.54

2.11 | Statistic

Statistical analysis was performed as described before42: All experiments were performed at least three times as indicated in the figure legends. Quantitative data are always presented as mean with standard deviation (SD). Statistical analyses were performed utilising the generalised mixed model analysis (PROC GLIMMIX, SAS 9.4, SAS Institute Inc., Cary, North Carolina, USA) and assumed to be from either normal, lognormal or beta distribution with the day of experiment conduction as random to assess differences in the size of treatment effects across the results. Residual analysis and the Shapiro-Wilk test were used as diagnostics. In the case of heteroscedasticity (according to the covtest statement) the degrees of freedom were adjusted by the Kenward-Roger approximation. All p-values were adjusted for multiple comparisons by the false discovery rate (FDR). p < .05 was considered significant with *p < .05, **p < .01.

3 | RESULTS

3.1 | Differential ACE2 expressions in tissues underline importance of soluble ACE2

The release of soluble and functional ACE2 may be of particular interest for tissues with low ACE2 expression, where circulating ACE2 can exert its function in place of membrane-bound ACE2. We analysed mRNA expression...
levels of ACE2 in different human tissues by utilising transcriptome data from healthy human samples from public repositories (File S1). Most tissues indeed show extremely low ACE2 expression levels including the lung, which is in line with an earlier report. In contrast, only the small intestine and the kidney show high ACE2 gene expression (File S1). This is in line with previous reports that the kidney is a main source of soluble ACE2. Thus, understanding the ACE2 shedding mechanism and subsequent formation of sACE2 is crucial for gaining insight into ACE2-dependent (patho)physiological processes. Noteworthy, our study revealed that in the tissues with increased ACE2 expression, the mRNA expression of ADAM10 is higher than that of ADAM17 (File S1), which is so far believed to be the predominant sheddase for ACE2.

### 3.2 ADAM10 is a constitutive ACE2 sheddase

ACE2 gene expression is high in kidney tissue (File S1) and the kidney seems to be a main source of soluble ACE2. Additionally, it is known that there is activity of mature ADAM10 in cells of the intestine, the kidney and the lung, where ACE2 plays a vital role.18,20,56,57 Additionally, we verified the presence of mature ADAM10 in these tissues on the protein level (Figure S1A). In the following experiments we used the human embryonic kidney cell line HEK293, which was described to have epithelial and mesenchymal characteristics. This cell line served as model system for most of the previous analyses on the molecular mechanisms of ADAM17-mediated ACE2 cleavage as well as for studies on SARS-CoV infection. To quantitatively investigate ACE2 shedding, we designed an ACE2 construct fused to an alkaline phosphatase (AP-ACE2) (Figure 1A), as shown before with other ADAM substrates. We transiently transfected HEK293 cells with AP-ACE2 and analysed constitutive ACE2 release over 24 h. ACE2 was released into the supernatant and this could be blocked by the inhibitor TAPI1 (Figure 1B), as previously described. However, TAPI1 is not specific for ADAM17 and has already been described as an effective inhibitor of ADAM10.31

In fact, ACE2 shedding was also reduced by the broad-spectrum metalloprotease inhibitor marimastat (Ma) and GI254023X (GI) (Figure 1B), which is 100-fold more potent inhibitor for ADAM10 than for ADAM17. We additionally found that ACE2-release is also reduced by GI in the intestine-derived epithelial cell line TC-7 (Figure S1B). Furthermore, the strong ADAM17 activator phorbol 12-myristate 13-acetate (PMA), significantly increases long-term ACE2 release within 24 h of stimulation (Figure 1C). This increased release is in turn inhibitable by TAPI1, Ma and GI (Figure 1C).

We further verified the involvement of both ADAMs as ACE2 sheddases using our CRISPR/Cas9-derived HEK293 cells deficient for ADAM17, ADAM10 or both. In these cells, as expected, the ADAM17 substrate TGFα is not cleaved in the absence of ADAM17, whereas BTC is not cleaved in the absence of ADAM10 (Figure S1C). Interestingly, we could not observe a reduction in ACE2 release in ADAM17-deficient cells (Figure 1D). In contrast, cells deficient for ADAM10 show a significant decrease in ACE2 release (Figure 1E,F). Additionally, in ADAM17-deficient cells, ACE2 release can still be blocked by TAPI1, Ma and GI (Figure S1D). Together these results clearly point to ADAM10 as a novel ACE2 sheddase. In contrast, ACE2 release in ADAM10-deficient cells cannot be reduced by GI (Figure S1E), also highlighting the specificity of the inhibitor against ADAM10 over ADAM17. In these cells, TAPI1 and Ma still decrease ACE2 release (Figure S1E), indicating still the involvement of ADAM17. In cells lacking both ADAMs ACE2 release is not sensitive to any of the used inhibitors (Figure S1F). Overall, these results demonstrate for the first time an involvement of ADAM10 in constitutive ACE2 release.

### 3.3 ACE2 release is enhanced by the ADAM10 activator ionomycin

Having observed involvement of ADAM10 in long-term ACE2 release, we also analysed short-term release (2 h). For a more homogenous ACE2 expression resulting in a better yield of soluble ACE2, HEK293 cells stably expressing AP-ACE2 were used (Figure S2A). We confirmed that these cells show the same TAPI1- and GI-sensitive long-term ACE2 shedding as the transiently transfected cells (Figure 2A). Further inhibition kinetics indicated that the IC50 for GI was about 15 nM. In comparison, the IC50 for TAPI1 and Ma were 1500 nM and 250 nM, respectively (Figure S2B,C,D), demonstrating again that ADAM10 seems to be a major sheddase for constitutive ACE2 shedding.

Additionally, we observed constitutive ACE2 release within 2 h, which can be reduced again by TAPI1 and GI (Figure 2B). We also attempted to stimulate short-term ADAM17-mediated ACE2 release with PMA. However, we did not observe any additional increase of ACE2 release compared with the control (Figure 2B). Instead, ACE2 release was no longer inhibitable by GI, but was still inhibitable by TAPI1, indicating that there was a shift from ADAM10 (loss of ADAM10-mediated ACE2 shedding) to ADAM17 activity (stimulation of ADAM17-mediated ACE2 shedding) with PMA treatment. This
FIGURE 2  ADAM10- and ADAM17-mediated ACE2 shedding can be induced. (A–C) HEK293 cells stably expressing 4xmycAP-ACE2 were used to analyse release via AP assay. Treatment as labelled: 100 nM PMA; 1 µM ionomycin (Iono); 10 µM TAPI1; 10 µM GI, and incubated for 24 h (A) or 2 h (B and C). n ≥ 4. (D) HEK293 cells stably expressing 4xmycAP-ACE2 or GFP as negative control (ctr.) were used to isolate extracellular vesicles (e.v.). Isolated e.v. and lysates were immunoblotted and analysed for AP-ACE2, HSP70 and GAPDH. Quality of e.v. isolation was evaluated by quantifying the ratio of GAPDH and HSP70 signals. n ≥ 3. (E–H) HEK293 cells (E and F) or HEK293 cells stably expressing iRhom2 (G and H), which were transfected with ACE2, were used to analyse proteolytic activity of soluble ACE2 via fluorogenic peptide cleavage assay. Cells were treated as labelled: 100 nM PMA; 40 µM TAPI1; 10 µM GI, and incubated for 2 h (E, F, and H). n = 3. (G) Stable expression of iRhom2 with HA-tag was analysed by immunoblotting. ADAM17 maturation is represented by the comparison of mature (mADAM17) with immature ADAM17 (imADAM17). The transferrin receptor (TfR) served as input control. Quantitative analysis of maturation is shown in Figure S3G. n = 4
is consistent with the model that short-term ADAM17 activity can be induced by PKC activation such as PMA treatment, whereas ADAM10 activation depends on Ca\textsuperscript{2+} influx.\textsuperscript{28,34–36} Moreover, Ca\textsuperscript{2+} influx can be inhibited by PKC activity.\textsuperscript{60,61} Consequently, PMA can decrease ADAM10 activity and increase ADAM17 activity, which would explain the observed shift. To support this assumption, we used the pan-PKC inhibitor BIMII. In PMA stimulated cells, neither BIMII nor the ADAM10 inhibitor GI alone could inhibit ACE2 shedding significantly. However, in combination both inhibitors significantly reduced the shedding, indicating that ADAM10 contributes to the shedding when PKCs are inhibited (Figure S2E).

Additionally, stimulation with the ADAM10 activator ionomycin, which leads to Ca\textsuperscript{2+} influx, resulted in shedding, indicating that ADAM10 contributes to the shedding when PKCs are inhibited (Figure S2E). In PMA stimulated cells, neither BIMII nor the ADAM10 inhibitor GI alone could inhibit ACE2 shedding significantly. However, in combination both inhibitors significantly reduced the shedding, indicating that ADAM10 contributes to the shedding when PKCs are inhibited (Figure S2E).

3.4 | ACE2 is released by extracellular vesicles

While ADAM10- and ADAM17-mediated shedding can be efficiently blocked by Ma, TAPI1 and GI, ACE2 release was only reduced by up to 50% (Figures 1B,C and 2A–C). Since the rhomboid protease RHBDL2 also shares substrates with ADAM17 and ADAM10 such as the IL6 receptor and IL11 receptor,\textsuperscript{49,52} we additionally tested this protease for its ability to shed ACE2. However, we found that overexpression of RHBDL2 and closely related members of the rhomboid superfamily have no influence on ACE2 release (Figure S2F). While there may still be other ACE2 sheddases, it was recently described that ACE2 can also be released via extracellular vesicles.\textsuperscript{63,64} To test this, we purified extracellular vesicles from HEK293 cells expressing AP-ACE2. Indeed, AP-ACE2 was detected in extracellular vesicles (Figure 2D). As control of purity of the isolated extracellular vesicles the exosomal marker HSP70 was used, which is enriched in extracellular vesicles compared to GAPDH (Figure 2D). In summary, sACE2 is not only released by ADAM10- and ADAM17-mediated shedding but also in extracellular vesicles.

3.5 | Shed ACE2 is catalytically active

Since circulating soluble ACE2 has been proven to be catalytically active in humans,\textsuperscript{9} we investigated whether sACE2 released by shedding retains its proteolytic activity. For this purpose, we measured the cleavage of an ACE2 peptide substrate. Indeed, shed ACE2 was catalytically active, and its release could be reduced by TAPI1 and GI (Figure 2E). Furthermore, we did not observe any additional release of active ACE2 after short-term stimulation with PMA (Figure 2F). This is in line with our finding that there is no additional release of ACE2 after short-term stimulation with PMA. Furthermore, we tested whether ACE2 is proteolytically active in extracellular vesicles using the ACE2 peptide substrate assay. We found that isolated extracellular vesicles from HEK293 cells overexpressing ACE2 showed proteolytic ACE2 activity (Figure S2G).

3.6 | iRhom2 expression facilitates a switch to ADAM17-mediated ACE2 shedding

As previously reported iRhom2 is a crucial promoter of ADAM17 activity under pro-inflammatory conditions.\textsuperscript{38,39,65,66} Thus, we hypothesised that iRhom2-dependent increase in ADAM17 activity increases ACE2 shedding.

We used HEK293 cells stably expressing iRhom2, as described before.\textsuperscript{42} Stable expression of iRhom2 increased the level of mature ADAM17 significantly (Figure 2G; Figure S3A,C), which subsequently increased ADAM17 expression on the cell surface (Figure S3B). Noteworthy, in iRhom2 overexpressing cells, the release of active ACE2 was significantly reduced by TAPI1 but no longer by GI (Figure 2H). Importantly, the release of active ACE2 in iRhom2 overexpressing cells was stimulated by PMA, which was not the case in cells without iRhom2 overexpression (Figure 2F,H). This stimulated release was significantly blocked by TAPI1 and only to a lesser extent by GI (Figure 2H). These results indicate, that ADAM10 is the major ACE2 sheddase for constitutive release under homeostatic conditions, whereas overexpression of the pro-inflammatory iRhom2 increases ADAM17-dependent ACE2 shedding.

3.7 | The collectrin-like part of ACE2 is sufficient for shedding

ACE2 consists of the N-terminal MD and the C-terminal CLP (Figure 3B). In general, ADAM10 and ADAM17 cleave proximal to the membrane within stalk regions of their substrates. Hence, it would be expected that the CLP is a main determinant for the shedding process. However, previously Jia et al.\textsuperscript{13} have described that the MD but not the CLP (Figure 3B) is necessary for shedding. They assumed the start of the CLP to be at R621.
We bioinformatically analysed the sequence of ACE2 and collectrin. We used secondary structure prediction and alignments to compare both sequences (Figure 3A). We found that the CLP of ACE2 is highly conserved to collectrin. The high conservation starts at K619 indicating that this is the first amino acid of the collectrin-like domain (CLD) (Figure 3B), which together with the stalk region, the transmembrane helix and a cytosolic tail represents the CLP. There is a short sequence between the predicted borders of the MD and the CLD. Since no secondary structure was predicted within this region, we assumed that this may be a short linker region (Figure 3A,B).

Based on our in silico determination of the domain boundaries, we designed a construct consisting only of the CLP starting at K619, comprising the CLD, the stalk region with the putative cleavage site(s), the TMH and the cytosolic tail, and fused it N-terminally to AP (Figure 4A). Long-term (24 h) release of soluble CLP was
detectable, which can again be blocked by Ma, TAPI1 as well as GI (Figure 4B). Moreover, the release of CLP is increased after stimulation with PMA, which is also inhibitable by the different used metalloprotease inhibitors (Figure 4B). In cells deficient for ADAM17 there is still GI- and TAPI1-inhibitable shedding (Figure S3D), which is completely lost in cells deficient for both ADAM17 and ADAM10 (Figure S3D). Interestingly, already the loss of only ADAM10 seems to prevent TAPI1 and GI-sensitive shedding (Figure S3D), again indicating that ADAM10 seems to be the more relevant sheddase in this instant.

Cells stably expressing CLP show similar results to transient expression in long-term shedding. (Figure 4B,C). Short-term release of CLP, which was also blockable by GI and TAPI1, was also detected in these stably expressing cells (Figure 4D). To analyse whether ADAM17 is still able to shed the CLP, we also generated HEK293 cells deficient for ADAM10, ADAM17 or both stably expressing AP-CLP. While no inhibitable shedding could be detected in cells lacking both ADAMs, ADAM17-deficient and ADAM10-deficient cells continue to show GI- and TAPI1-blockable shedding, respectively (Figure S3E).

Short-term release of AP-CLP can also be additionally stimulated by the ADAM10 stimulator ionomycin and can be decreased by TAPI1 and GI (Figure 4D). Interestingly, in contrast to full-length ACE2 (Figure 2B), CLP short-term release can be increased by PMA and can be blocked by GI and TAPI1. This could indicate that the CLP alone is generally more susceptible to the shedding process.

**FIGURE 4** The CLP of ACE2 is susceptible to shedding. (A) CLP was N-terminally fused with AP (AP-CLP). (B–D) HEK293 cells transiently (B) or stably (C and D) expressing AP-CLP or 4xmycAP-CLP, respectively, were used. Cells were treated as labelled: 100 nM PMA; 1 µM ionomycin (Iono); 40 µM TAPI1 (B), 10 µM TAPI1 (C and D); 10 µM GI; 25 µM Ma, and incubated for 24 h (B and C) or 2 h (D) n ≥ 3.
In summary, we could for the first time demonstrate that the CLP of ACE2 alone is sufficient for the ADAM10- and ADAM17-mediated shedding process.

4 | DISCUSSION

ACE2 is a central player in the RAS by counterbalancing the activity of ACE and thereby promoting physiological processes such as vasodilation, anti-fibrotic actions and anti-inflammatory effects (Figure 5). On the other side it is as the receptor of SARS-CoV-1 and -2 critically involved in virus infection and hence an important target to overcome the recent health crisis (Figure 5). Interestingly, we and others found that ACE2 is only low expressed in most human tissue including the lung but is highly expressed in the human kidney (File S1). Released sACE2 that circulates through the body and is still active may act in a distal manner in the RAS (Figure 5).

A currently more pressing involvement of ACE2 shedding would be its putative effect on infections with SARS-CoV-1 and the various current and future strains of SARS-CoV-2. Here, shedding ACE2 as a SARS-CoV receptor from the cell surface would potentially promote protection from virus binding and thus infection. In addition, soluble ACE2 ectodomains could act as scavenger receptors and bind the spike protein of the virus particles. This would prevent the virus particles from binding to cell-surface ACE2. This process, which would also block viral infection, has been described previously.\(^{16,63,67}\) Hence a proposed strategy to counter SARS-CoV infections is the use of recombinant ACE2 derivates.\(^{16,67-69}\) However, soluble ACE2 may have also a negative effect on SARS-CoV-2 progression in patients.\(^{70}\) Additionally, it was recently described that soluble ACE2 may actually facilitate SARS-CoV-2 infections.\(^{17}\) Finally, in the vascular system the enzymatic activity of released ACE2 may influence development of COVID19. Further studies are needed to gain more insight in either protective or detrimental roles of ACE2 cleavage during the course of the disease. Initially, before the contact with the virus constitutive shedding by ADAM10 might be more relevant to provide soluble ACE2. Later ADAM17-mediated shedding could be stimulated by inflammatory conditions and, as previously described, by interaction with the spike protein of SARS-CoV-1, leading to more soluble ACE2.\(^{71}\)

It is also worth noting that these mechanisms might not only be relevant for SARS-CoV-1 and SARS-CoV-2, but also for others (corona)viruses that might use ACE2 as an entry receptor. So far, however, only the coronavirus NL63 has been found that appears to bind to ACE2, but with less affinity than SARS-CoV.\(^{72}\)

Previously, it was demonstrated that ADAM17 constitutively sheds ACE2,\(^{7,13,15}\) which we could also confirm in this study. It is known that ADAM17 and ADAM10 share substrates such as IL6R,\(^{73}\) CX3CL1\(^{74,75}\) and Notch1.\(^{76,77}\) With this study, we highlight that this is also true for ACE2 and that ADAM10 is a major ACE2 sheddase. We could
also demonstrate that sACE2 derived from ADAM10- or ADAM17-mediated shedding is proteolytically active and that ACE2 is additionally released in extracellular vesicles.

It is known that ADAM10 is mainly a constitutively active sheddase, while ADAM17-mediated shedding often requires stimulation. Based on our results, the primary sheddase of ACE2 seems to be ADAM10, while ADAM17 seems to play only a minor role even after stimulation (Figure 5). We found that ADAM10-mediated shedding of ACE2 can be further upregulated by stimulation with ionomycin. This is in line with reports showing that stimulation of ion channels can enhance ADAM10 activity.31,78 Interestingly, it was reported that ACE2 shedding by ADAM17 is also stimulated during infection with SARS-CoV-1.71 Furthermore, it was reported that sACE2 levels are elevated in different pathologies.9 Noteworthy, ADAM17 activity is also upregulated after inflammatory stimuli. Here, not the upregulation of ADAM17 mRNA or protein levels are crucial but the upregulation of iRhom2 as an important ADAM17 interactor and regulator.38,40,65,79,80 Under physiological conditions iRhom2 is mainly expressed in immune cells (File S1). However, we recently found that iRhom2 can also be induced in other cells such as endothelial cells under inflammatory conditions causing elevated ADAM17 activity.38 In this study we demonstrated that there is a switch to more ADAM17-dependent ACE2 shedding when iRhom2 is overexpressed (Figure 5), which mimics ADAM17 activity under inflammatory conditions.38,40,65,66,79,81

Previously, it was reported that the main structural determinant for ADAM17-mediated ACE2 shedding is not the CLP but the MD of ACE2.13 Noteworthy, Jia et al.13 defined the start of the CLP at R621 in their used construct. However, our secondary structure prediction demonstrated that this domain boundary determination would destroy a putative β-sheet (Figure 3A). This may possibly cause misfolding and explain the reported absence of shedding of this partial CLP. We determined via our in silico analyses that the CLP domain should start at K619. The recently solved structure of the whole ACE2 ectodomain47 confirmed this (Figure 3B). In fact, we demonstrated that this complete CLP was well shed by ADAM10 and ADAM17. Moreover, we showed that the CLP without the MD seems to be even more susceptible to PMA-induced short-term shedding than full length ACE2. We can just speculate that this may be due to the loss of steric hindrance by the MD. Interestingly, Heurich et al.14 tried to determine the cleavage sites of ADAM17 and TMPRSS2 by mutating different clusters. However, as all these clusters are located within the structured parts of the CLD, these constructs will most likely affect the structural integrity of the CLD and should therefore be interpreted with caution.

Furthermore, the ACE2 parologue collectrin(TMEM27) is described as a necessary chaperone/trafficking partner of the amino acid transporter B0AT1 in the kidney, while ACE2 assumes this function in the small intestine, where collectrin expression is low.82,83 Without either collectrin or ACE2 expression B0AT1 is neither detectable at the cell membrane nor can amino acid uptake take place. While the importance of ACE2 for B0AT1 in the small intestine is clear, it is not yet known if there is any effect on ACE2 functions when the ACE2-B0AT complex is formed. Vice versa, it has been reported that B0AT1 is neither necessary for ACE2 cell surface expression nor SARS-CoV-1 or SARS-CoV-2 infection.4,5,14 However, this complex was still considered during the design of BNT162b vaccines against SARS-CoV-2.84 It was recently described that the heterodimer consisting of ACE2 and B0AT1 forms a tetramer consisting of two heterodimers.47,85 It is not known what effects B0AT1 has on the functions of ACE2, nor what the interaction dynamics of the ACE2-B0AT1 complex are. However, previous in silico data suggest that it is a steric hindrance for sheddases such as ADAM17 to reach the ACE2 stalk region with the putative cleavage sites when ACE2 is in complex with B0AT1, especially if it is a tetrameric formation.47,85–87 As this has implications for the ACE2 shedding event, especially in the intestine where B0AT1 expression is high, this needs to be thoroughly analysed in future experiments.

Overall, we provide evidence that ACE2 is released via ADAM10- and ADAM17-mediated shedding (Figure 5) as well as in extracellular vesicles and that for the shedding process only the CLP is sufficient. There might be further sources of sACE2 which should be analysed in further studies. It will also be crucial to define the cleavage site(s). Even more importantly it should be analysed which form of ACE2 release is of either physiological or pathophysiological relevance for ACE2 biology and whether different forms of soluble ACE2 have different (patho)physiological consequences.

During the SARS-CoV-2 outbreak, it has been heavily debated whether the ADAM17-ACE2 axis can be targeted to protect against viral infection.38 On the one hand, it is assumed that the increase in ADAM17-mediated shedding has a protective effect.89,90 On the other hand, it has been shown that ADAM17 activity itself may facilitate viral entry.71,91,92 Therefore, it is of crucial interest to consider alternative forms of soluble ACE2 release with parallel deletion of ACE2 at the cell surface, e.g. release via extracellular vesicles or ADAM10-mediated shedding, in future therapeutic strategies.

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The authors declare no competing interests.

AUTHOR CONTRIBUTIONS
Stefan Düsterhöft and Andreas Ludwig conceived, designed and coordinated the study. Stefan Düsterhöft, Andreas Ludwig and Rabea Victoria Niehues wrote the manuscript. Stefan Düsterhöft, Andreas Ludwig and Rabea Victoria Niehues analysed data and revised the manuscript. Stefan Düsterhöft performed in silico analysis. Rabea Victoria Niehues, Justyna Wozniak, Eva Lilienthal, Florian Wiersch, Christoph Garbers, Tim Schumertl and Nikola Tacken performed experiments and analysed the results. All authors read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT
For gene expression data analysis the Genevestigator database was accessed by using the Genevestigator suite (https://genevestigator.com).

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