The receptor for advanced glycation end products (RAGE) is a 55-kDa type I membrane glycoprotein of the immunoglobulin superfamily. Ligand-induced up-regulation of RAGE is involved in various pathophysiological processes, including late diabetic complications and Alzheimer disease. Application of recombinant soluble RAGE has been shown to block RAGE-mediated pathophysiological conditions. After expression of full-length RAGE in HEK cells we identified a 48-kDa soluble RAGE form (sRAGE) in the culture medium. This variant of RAGE is smaller than a 51-kDa soluble version derived from alternative splicing. The release of sRAGE can be induced by the phorbol ester PMA and the calcium ionophore calcimycin via calcium-dependent protein kinase C-secretase inhibitor, suggesting that RAGE-CTF is normally further processed by γ-secretase inhibitor, suggesting that RAGE-CTF is normally further processed by γ-secretase. Identification of these novel mechanisms involved in regulating the availability of cell surface-located RAGE and its soluble ectodomain may influence further research in RAGE-mediated processes in cell biology and pathophysiology.

Receptor for advanced glycation end products (RAGE) is expressed in a variety of tissues and cells, including endothelial cells, vascular smooth muscle cells, and neurons. Structurally, RAGE is composed of an extracellular region containing one V-type and two C-type immunoglobulin domains, a single hydrophobic transmembrane domain, and a short cytoplasmic tail, which is essential for RAGE signaling (1). An involvement of RAGE in various pathophysiological processes such as chronic inflammatory diseases, late diabetic complications, atherosclerosis, and Alzheimer disease has been demonstrated (2). Proteins and peptides like advanced glycation end products, Alzheimer amyloid β-peptides (Aβ), S100/calgranulin family members, and HMGB1 (amphoterin) have been identified as ligands for RAGE (3). Because of the diversity of the RAGE ligands, it is assumed that RAGE is a pattern recognition receptor (4, 5).

RAGE was also identified as the human blood-brain barrier receptor for Aβ (6) responsible for the uptake of serum Aβ into the central nervous system (7). In addition, by application of recombinant soluble RAGE, which comprises the extracellular ligand binding domain, the accumulation of Aβ in brain parenchyma of transgenic APP mice could be suppressed (7). Recombinant sRAGE generally seems to be able to reduce or even reverse RAGE-mediated pathological symptoms: it prevents diabetic atherosclerosis (8) and inhibits tumor metastasis and invasion (9).

Native forms of sRAGE have been found in circulating blood (10) as well as in tissues such as brain, lung, and tumor cells (11, 12). Several groups have shown that soluble RAGE derives from alternative splicing of the RAGE mRNA (11–13). The primary secreted splice variant of human RAGE is esRAGE (endogenous secretory RAGE, also named RAGE_v1 (14)), and also in mouse a secreted homologue of esRAGE was identified (15). Both esRAGE variants contain nearly the entire extracellular part of full-length RAGE, including the V-type ligand binding domain. In human lung 80% of the RAGE mRNA encodes for full-length RAGE and only 7% for esRAGE (14). Nevertheless, in human lung samples the full-length membrane-bound RAGE protein is difficult to detect, whereas the majority of the RAGE protein is found in a soluble form (16). Therefore, mechanistic studies are necessary to determine the biological role of sRAGE.
anisms different from alternative splicing may account for the secretion of RAGE.

Because the amount of membrane-bound RAGE is increased in the brain vasculature of AD patients, we hypothesized that besides an increased expression also a reduced proteolysis of RAGE may be responsible for the presence of cellular RAGE. In this report we demonstrate that the ligand binding domain of RAGE is proteolytically released from membrane-bound RAGE by a metalloproteinase, and we identified calcium-dependent PKC isoenzymes to be involved in activation of that process. Furthermore, we show that after ectodomain shedding RAGE is subsequently cleaved by γ-secretase.

**Experimental Procedures**

**Antibodies and Reagents**—Anti human RAGE N-terminal antibody Mab5328 (Millipore) was used for detection of human RAGE. Rabbit serum Ab3260, which recognizes soluble human RAGE, was made at Eurogentec (Seraing, Belgium). For immunization the RAGE peptide sequence VSISIIEPGEEGPTAG (Val-316 to Gly-331 of human RAGE) was coupled to keyhole limpet hemocyanin.

Mouse monoclonal anti-myc antibody 9E10 (hybridoma cell culture supernatant) and an anti V5 antibody (Invitrogen) were used to detect N-terminally myc- and C-terminally V5-tagged human RAGE. The anti-actin antibody A2066 was from Sigma-Aldrich. Human ADAM10 and ADAM17 proteins were detected with antibodies from Millipore.

Secondary anti-mouse and anti-rabbit antibodies either peroxidase-coupled or 35S-labeled were from GE Healthcare Life Sciences (Freiburg, Germany). ECL detection reagent was from Perbio Science; all other chemicals were from Merck Biosciences (Bad Schwalbach, Germany) and Roche Diagnostics (Mannheim, Germany).

**Expression Vectors**—The human RAGE cDNA clone IRALp962E1737Q2 was from RZPD (Berlin, Germany). With DNA from clone IRALp962E1737Q2 as template, PCR mutagenesis was performed to insert a myc tag coding sequence 3’ after the signal peptide coding region and a SacII site 5’ to the stop codon of human RAGE, the PCR product was inserted between KpnI and SacII sites of pcDNA6/V5-HisB (Invitrogen), thus creating a myc-RAGE-V5-His (tgRAGE)-expressing construct. To generate the untagged wild-type human RAGE expression vector pcDNA6-RAGE the human RAGE cDNA, including its own translation termination codon, was subcloned from IRALp962E1737Q2 into EcoRI/XbaI sites of pcDNA6/V5-HisB. Mutated RAGE cDNAs were made by standard PCR mutagenesis.

**Transfection of Cells**—HEK Flp-In™ 293 cells (Invitrogen) were transfected with pcDNA6 RAGE expression plasmids using Lipofectamine 2000 (Invitrogen) and selected with blasticidin to generate stable cell lines expressing N-terminally Myc-tagged and C-terminally V5-His-tagged RAGE (HEK-tgRAGE cells) as well as wild-type human RAGE (HEK-RAGE) and mutated RAGE.

**Small Interference RNA Experiments**—Stealth RNAi duplexes were purchased from Invitrogen, and transfections were performed according the manufacturer’s protocol. The RAGE cleavage assay, gelatin zymography, and Western blot analysis were performed 48 h after transfection.

**RAGE Cleavage Assays and Inhibitor Treatments**—RAGE-expressing cells were plated on poly-L-lysine-coated 6-well plates and grown for 24 h to 80–90% confluence. Cells were washed twice with serum-free DMEM, and then secretion medium (SM, serum-free DMEM supplemented with 2 mM glutamine and chemicals of interest) was added.

Sheding inducers like PMA or Calcimycin (A23187) were added to the secretion medium (SM) directly at the beginning of the incubation time. If experiments were performed in the presence of inhibitors of proteinases, intracellular protein transport or PKC, cells were preincubated in SM containing the respective substances for 1 h at 37 °C to secure uptake of the compounds. Afterward this medium was replaced by fresh SM containing the substance of interest together with the inhibitor. The concentrations of the compounds used in the experiments are given in the results part. Following an appropriate incubation period (indicated in the text and the figures) cell culture supernatant was collected and centrifuged for 10 min at 660 × g to remove residual cells. Proteins in the cleared supernatants were precipitated with 10% trichloroacetic acid at 4 °C and dissolved in reducing Laemmli buffer. Always the whole amount of precipitated proteins was used for Western blot analysis (see below). For comparative and quantitative analysis, effects observed with DMSO-treated cells were used as control and were set to 100%.

Adherent cells on the coated plates were washed with PBS and dissolved in reducing Laemmli buffer. The protein content of cell lysates was determined by the Bradford method after chloroform/methanol precipitation of sample aliquots. For Western blot analysis of cell lysates always 20 μg of protein was used.

**Deglycosylation Assay**—Serum-free cell culture supernatant of RAGE-expressing cells was collected from 10-cm plates as described above. After trichloroacetic acid precipitation, the proteins were dissolved in 5% SDS containing 10% β-mercaptoethanol and incubated for 10 min at 100 °C. Subsequently, the protein solution was diluted with 9 volumes of H2O and equally divided into two aliquots. One aliquot was treated with PNGaseF (New England Biolabs, Frankfurt, Germany), which removes nearly all types of N-glycan chains from glycoproteins, according to the manufacturer’s protocol. The second aliquot was treated equally but without applying PNGaseF. Finally proteins were precipitated again with trichloroacetic acid, dissolved in reducing Laemmli buffer, and analyzed by Western blotting. Deglycosylation of cell-bound full-length RAGE was performed similarly with cell lysates as starting material.

**Biotinylation of Cell Surface Proteins**—Adherent cells were washed twice with PBS and then incubated for protein biotinylation with PBS containing 0.1 mM Sulfo-NHS-LC-Biotin (Pierce Biotechnology) for 30 min at room temperature. After washing with Tris-buffered saline (pH 7.4) the cells were incubated in secretion medium for different periods as indicated in the results part. The cells and supernatants were collected separately, and the supernatants were adjusted to 0.1% SDS. The cells were dissolved in 5% SDS, and then SDS was diluted to 0.1% by adding PBS. Biotinylated proteins were captured with
NeutrAvidin Biotin-binding agarose (Pierce) for 2 h at 4 °C. After that the agarose beads were washed three times with PBS/0.1% SDS. Proteins were eluted from the binding agarose with reducing Laemmli buffer and analyzed by Western blotting. Actin could not be detected in isolated biotinylated protein fractions, thus demonstrating the integrity of the cells and the biotinylation reagent during the course of the experiment. As a control, actin could be detected in the cell lysate (5% SDS fraction; see above).

**Western Blotting**—Proteins of the cell culture supernatant or cells lysates were separated either by 10% SDS-PAGE or NuPAGE gradient gel (Invitrogen) electrophoresis and then electroblotted to nitrocellulose membranes (GE Healthcare). Membranes were probed with respective primary and secondary antibodies. Labeled proteins were detected and quantified by chemiluminescence or phosphorimaging using the VersaDoc system (Bio-Rad Laboratories, Munich, Germany) or the Bio-Imaging Analyzer BAS-1800 (Fujifilm Medical Systems, Düsseldorf, Germany). Expression of the housekeeping protein actin was routinely determined to confirm equal loading of proteins.

**Gelatin Zymography**—Cells were washed twice with serum-free DMEM, then SM supplemented with either PMA (1 μM) or DMSO was added to the cells. After 18 h of incubation at 37 °C, supernatants were collected and cleared from cells by centrifugation (660 × g, 10 min). For gelatin zymography 30 μl of serum-free cell culture supernatant was mixed with 10 μl of 4× NuPAGE LDS Sample Buffer (Invitrogen), and subsequently proteins were separated under non-reducing conditions in 10% NuPAGE LDS Sample Buffer (Invitrogen), and subsequently proteins were separated under non-reducing conditions in 10% SDS-polyacrylamide gels containing 0.1% gelatin. After electrophoresis gels were washed in 2.5% Triton X-100 at room temperature for 1 h and then incubated for 16 h at 37 °C in reaction buffer (100 mM Tris-HCl, pH 7.5, 10 mM CaCl2). Finally the gels were stained with Coomassie Blue. Clear areas within the stained gel result from digestion of gelatin by the gelatinase activity of either MMP9 or MMP2.

**RESULTS**

**Full-length RAGE-expressing Cells Secrete a Soluble Form of RAGE Smaller Than the Splice Variant “Endogenous Secretory RAGE”**—To investigate the putative proteolytic processing of human RAGE, we generated HEK cell lines stably expressing either wild-type human RAGE (HEK-RAGE cells) or esRAGE (HEK-esRAGE cells) with esRAGE being a recombinant version of the splice variant endogenous secretory RAGE (esRAGE).

In the cell culture supernatant of HEK-RAGE and HEK-esRAGE cells soluble proteins were detected with antibody Mab5328 directed against the N terminus of human RAGE. In the supernatant of full-length RAGE-expressing cells (HEK-RAGE) we found a major 48-kDa form of RAGE and a minor one of 45 kDa (Fig. 1A). Larger RAGE variants were identified to be secreted from HEK-esRAGE cells: a major form of 51 kDa and a minor of 49 kDa (Fig. 1A). After PNGaseF-mediated deglycosylation of secreted proteins in both cases smaller forms could be detected. In lysates of HEK-RAGE cells a double band at ~55 kDa corresponding to full-length RAGE was detected, and in lysates of HEK-esRAGE cells a diffuse band at ~51 kDa was present (Fig. 1A). After PNGaseF-mediated deglycosylation of full-length RAGE still a double band at ~53 kDa was detected (Fig. 1B). The occurrence of a double band is likely to be caused by different O-glycosylation and/or protein size, e.g. presence or absence of the signal peptide. Because RAGE secreted from full-length RAGE-expressing cells is smaller than endogenous secreted RAGE usually generated by alternative splicing, the data imply that sRAGE might be released by proteolysis of full-length RAGE.

**FIGURE 1. Detection of soluble RAGE forms in cell culture supernatant of RAGE-expressing cells.** RAGE was detected by Western blotting using antibody Mab5328 directed against the N-terminal region of human RAGE and ECL. RAGE, samples from HEK cells expressing the type I membrane protein full-length RAGE, esRAGE, samples from HEK cells expressing a soluble recombinantly produced splice form of RAGE; mock, sample of untransfected HEK cells. A, detection of soluble RAGE forms. Lane 1, proteins from cell culture medium of RAGE-expressing cells; lane 2, same as in lane 1 but after protein deglycosylation using PNGaseF; lane 3, proteins from cell culture medium of esRAGE-expressing cells; lane 4, same as in lane 2 but after protein deglycosylation using PNGaseF; lane 5, cell lysate of RAGE-HK cells; lane 6, cell lysate of esRAGE cells; lane 7, cell lysate of untransfected HEK cells. B, deglycosylation of full-length RAGE. RAGE in HEK-RAGE cells before (lane 1) and after (lane 2) PNGaseF-mediated deglycosylation.

The Release of Soluble RAGE from Full-length RAGE-expressing Cells Is Inducible and Mediated by Proteolysis—To distinguish between shedding and proteolysis-independent protein secretion, we compared the release of soluble RAGE among cells expressing either full-length membrane-bound RAGE or recombinant versions of soluble RAGE under different experimental conditions. If RAGE-expressing cells were treated with the organomercurial agent APMA, which enables latent matrix metalloproteinases to undergo autolytic self-activation (17), the release of RAGE was strongly induced exclusively in cells expressing full-length membrane-bound RAGE (Fig. 2A).

Treatment of cells with the phorbol ester PMA induced the release of soluble RAGE from cells expressing full-length RAGE, esRAGE, or recombinant sRAGE (sRAGE, composed of amino acids 1–331 of full-length RAGE).

PMA is a known inducer of shedding events, but it also enhances the release of soluble proteins via exocytosis in the secretory pathway (18). To differentiate induced shedding from enhanced exocytosis, we treated cells with compounds known to prevent vesicle-dependent protein secretion from the endoplasmic reticulum and the Golgi apparatus. In cells expressing recombinant soluble RAGE forms PMA slightly enhanced RAGE expression (Fig. 2, B and D), but both brefeldin A (10 μM) as well as monensin (2 μM) prevented the PMA-induced release of RAGE from these cells (Fig. 2A). This resulted in increased amounts of intracellular RAGE (Fig. 2B). In contrast, brefeldin A and monensin were not able to block the release of RAGE from cells expressing full-length RAGE suggesting that sRAGE is released from these cells by proteolysis of already membrane-
located RAGE and not by exocytosis of an intracellular sRAGE form. After PMA induction of full-length RAGE-expressing cells the secretion of RAGE was less pronounced when cells had been treated with brefeldin A (Fig. 2A, lane 5 versus 4). This observation can be explained by a reduced delivery of full-length RAGE from its site of biosynthesis in the endoplasmic reticulum to its site of proteolysis at the cell surface, because brefeldin A is known to block protein transport from the endoplasmic reticulum to the Golgi apparatus.

Treatment of RAGE-expressing cells with GM6001, a metalloproteinase inhibitor known to block shedding events, further supports this assumption. In full-length RAGE-expressing cells the release of RAGE was almost completely prevented by GM6001 (Fig. 2C), furthermore administration of GM6001 increased full-length RAGE levels (Fig. 2D). In contrast, the release of RAGE from cells expressing recombinant soluble RAGE forms was not affected by GM6001 (Fig. 2C). Treatment of full-length RAGE-expressing cells with either PMA or APMA always induced the release of the soluble 48-kDa RAGE form (Fig. 2A and C); the occurrence of a 51-kDa variant, corresponding to alternatively spliced esRAGE, was not observed. This result shows that both compounds did not induce an alternative splicing mechanism. Therefore, our data clearly demonstrate that full-length RAGE is subjected to protein ectodomain shedding.

When HEK-RAGE cells were treated with shedding inducers, an obvious difference became evident after either APMA or PMA stimulation. Incubation with the more potent proteinase activator APMA strongly induced the release of sRAGE (Fig. 2A) and in parallel decreased the amount of the full-length RAGE substrate (Fig. 2B). Such a clear substrate/product relationship was not observed after treatment with the shedding inducer PMA under the experimental conditions applied. Therefore, we further examined the effect of PMA under two different experimental conditions: To analyze whether RAGE located at the cell membrane is the substrate of proteolysis, proteins located at the plasma membrane were labeled with a membrane-impermeable biotinylation reagent before shedding was induced by PMA. Afterward the amounts of biotinylated secreted RAGE and biotinylated membrane-bound RAGE were quantified by Western blotting, as control unlabeled cells were treated equally.

Treatment of full-length RAGE-expressing cells with the phorbol ester PMA (1 μm for 2 h) strongly induced the release of the soluble 48-kDa RAGE form (Fig. 3A). Compared with unstimulated conditions the increase of sRAGE was usually 2.5- to 5-fold. Quantification of full-length RAGE in total cell lysates showed only a slight decrease of full-length RAGE (Fig. 3B, left). In contrast, a significant decrease (33%) of full-length RAGE in the plasma membrane was obvious after PMA treatment (Fig. 3B, right), when the subcellular portion of RAGE located on the cell surface was investigated after biotinylation of cell-membrane proteins. Quantification of actin in cell lysates demonstrates that samples with equal amounts of proteins were compared (Fig. 3C). The experiment with biotinylated proteins demonstrates that PMA stimulates the release of already cell-surface-located full-length RAGE by a shedding mechanism.

To verify our finding that the increased amount of secreted RAGE after PMA treatment is not caused by de novo gene expression, we investigated the proteolysis of RAGE in the presence of the protein biosynthesis inhibitor cycloheximide. The PMA-induced release of sRAGE was not affected after treatment of RAGE-expressing cells with cycloheximide. When compared with DMSO–treated cells, PMA induced a 3-fold increase in sRAGE in the presence of cycloheximide (Fig. 4); further supporting the hypothesis that sRAGE is
generated by proteolysis. Treatment with PMA did not alter the amount of RAGE in total cell lysates (Fig. 4).

To confirm that full-length RAGE is a substrate for proteolytic cleavage, we also investigated the presence of a membrane-anchored C-terminal fragment of RAGE, which should be generated simultaneously in addition to sRAGE. By using an anti-V5 antibody directed against the C terminus of epitope-tagged human RAGE (tgRAGE), we detected a ~13-kDa RAGE fragment next to full-length RAGE (~55 kDa) in cell lysates (Fig. 5A, lane 1). Furthermore, treatment with the shedding inducer PMA clearly increased the amount of the RAGE C-terminal fragment (Fig. 5A, lane 1 versus 3).

For a variety of proteins such as the amyloid precursor protein (APP) and the Notch receptor ectodomain shedding is known to be followed by a second proteolytic step by γ-secretase. This cleavage liberates the C-terminal intracellular part, which, at least in the case of Notch, modulates gene expression. To examine the possibility that ectodomain shedding of RAGE precedes further γ-secretase processing, we investigated the effect of γ-secretase inhibition on RAGE metabolism.

Compared with DMSO-treated cells, treatment of RAGE-expressing cells with the γ-secretase inhibitor DAPT increased the amount of the membrane-bound 13-kDa RAGE C-terminal fragment (RAGE-CTF) under unstimulated (Fig. 5A, lane 1 versus lane 2) and PMA-stimulated conditions (Fig. 5A, lane 3 versus lane 4). DAPT neither enhanced the expression of RAGE (Fig. 5A) nor stimulated its secretion (Fig. 5B, lane 1 versus lane 2 and lane 3 versus lane 4). Therefore, the larger amount of RAGE-CTF can most likely be assigned to its increased stability in the presence of the γ-secretase inhibitor. This indicates that RAGE-CTF generated by ectodomain shedding of full-length RAGE is a substrate for subsequent γ-secretase cleavage.

Human RAGE Can Be Released Efficiently from the Cell Surface—To analyze the efficiency of RAGE shedding, we investigated the proteolysis of biotinylated cell surface located full-length RAGE. The release of biotinylated RAGE was monitored after isolation of biotinylated proteins from the cell culture supernatant and Western blot analysis. In parallel, the amount of biotinylated full-length RAGE remaining on the cells was analyzed.

During a 5-h treatment with PMA (1 μM), a strong decrease in the amount of full-length RAGE was observed on the cell surface (Fig. 6A, lane 3 versus lanes 1 and 2). In parallel to this decrease, the amount of biotinylated shedded RAGE in the cell culture supernatant significantly increased during 5 h of PMA stimulation (Fig. 6A, lane 7 versus lanes 5 and 6). In addition, unstimulated cells demonstrated constitutive shedding of RAGE (Fig. 6A, lane 8 versus lanes 5).

APMA treatment efficiently enhanced the release of the ectodomain of biotinylated RAGE from cells; within a 15-min treatment ~75% of previously cell-membrane bound RAGE was secreted (Fig. 6B). APMA treatment (15 min) induced a much faster release of sRAGE than PMA treatment (2 h in this experiment), most likely caused by a direct APMA-dependent
activation of the RAGE sheddase. Both treatments reduced the amount of cell-bound RAGE to the same extent but in the supernatant of the APMA-treated sample more sRAGE was present compared with the PMA-treated sample (Fig. 6B). This indicates that sRAGE has been partially degraded during the 8-fold longer incubation time with PMA in the presence of the secreting cells. Taken together, the results demonstrate that RAGE can be removed very efficiently from the cell surface by proteolysis.

Localization of the RAGE Cleavage Site—As shown in Fig. 5 cleavage of full-length RAGE generated sRAGE and a RAGE-CTF of ~13 kDa suggesting that full-length RAGE (55 kDa) is cleaved in its ectodomain C-terminally to its Ig and major histocompatibility complex protein signature. Moreover, a soluble form of mouse RAGE was purified from lung and by protein sequencing the C-terminal residue of this protein was found to be Gly-330 within the sequence motif PEAG present in the proximal ectodomain of RAGE nine amino acids above the transmembrane domain (19). Because the C terminus of this soluble mouse RAGE variant is identical to the corresponding region of full-length mouse RAGE but differs from mouse esRAGE, it is presumably generated by proteolysis.

To study whether human RAGE is cleaved within the same region, a polyclonal antibody against the epitope 316–VSISILEP

**FIGURE 4.** PMA-induced secretion of RAGE in the presence of cycloheximide. Detection and quantitation of secreted RAGE (sRAGE) in cell culture supernatants of HEK-tgRAGE and untransfected control cells (mock). A, where indicated, cells were preincubated with 45 μg/ml (160 μM) cycloheximide (Cyclo.) for 1 h, then the medium was replaced by PMA (1 μM) and/or cycloheximide containing medium. After 1.5 h sRAGE from the cell culture supernatant was analyzed by Western blot analysis using anti-myc antibody 9E10 and ECL. Full-length RAGE (fl-RAGE) and actin were detected in cell lysates by individual Western blot analyses. B, quantitation of secreted RAGE (sRAGE). Experiments were performed in triplicates. Shown is the mean effect ± S.D. after 1.5 h of treatment with either 1 μM PMA or the solvent DMSO. Significance was determined by the Student's unpaired t test; *, p < 0.05; **, p < 0.01.

**FIGURE 5.** Detection of the RAGE C-terminal fragment in cell lysates. HEK-tgRAGE or untransfected control cells (mock) were treated for 1.5 h with either the γ-secretase inhibitor DAPT (2 μM) or an equal amount of DMSO, then cells were treated for 1.5 h with either 1 μM PMA or DMSO in the presence or absence of DAPT (2 μM). A, cells were lysed in reducing NuPAGE sample buffer and subjected to Western blot analysis using 4–12% NuPAGE gradient gels. For detection of the RAGE C-terminal fragment (RAGE-CTF) and full-length RAGE (fl-RAGE) an anti-V5 antibody directed against the C terminus of recombinant RAGE was used. Actin was detected by a separate Western blot analysis in the same cell lysates. B, detection of secreted RAGE in the cell culture supernatant of treated cells by Western blot analysis using Mab5328.
RAGE Shedding Is Mediated by Zinc-dependent Metalloproteinases—Phorbol ester-inducible protein ectodomain shedding is known to be mediated by zinc-dependent metalloproteinases (20). Because RAGE shedding could be induced with the phorbol ester PMA and inhibited with the hydroxamic acid-based zinc-metalloproteinase inhibitor GM6001 (Fig. 2C) we quantified the influence of metalloproteinase inhibition on RAGE shedding. Application of GM6001 (10 μM) significantly decreased constitutive cleavage of RAGE by ~28% and PMA-induced shedding by ~36% (Fig. 8B). Other hydroxamic acid-based inhibitors had similar effects (data not shown). Inhibition of cysteine proteinases with E-64 (1.4 μM), aspartic proteinases by pepstatin (1.4 μM), and serine proteinases by Pefabloc SC (1 mM) revealed no significant inhibition of RAGE shedding (data not shown). Thus, RAGE is cleaved in its ectodomain by a zinc-dependent metalloproteinase, most likely belonging to the large families of disintegrin metalloproteinases (ADAMs) and matrix metalloproteinases (MMPs) (21).

Next we investigated whether ubiquitously expressed ADAM and MMP family members contribute to the shedding of RAGE. ADAM10 and ADAM17 were chosen because they are known sheddases for a variety of substrates (22). MMP9 and MMP2 were selected because they have been implicated in protein ectodomain shedding (23). The contribution of these proteinases on endogenous RAGE shedding was studied after RNAi-mediated knockdown of each family member. In all cases the knockdowns were very efficient as shown by a MMP2/9 gelatinase assay (Fig. 9A) and Western blot analysis of ADAM10 and ADAM17 expression (Fig. 9B). A reduction in constitutive and PMA-induced shedding of RAGE was evident when either ADAM10 or MMP9 were depleted (Fig. 9A). The knockdowns of ADAM17 and MMP2 had no obvious effect on RAGE shedding (Fig. 9A), although both proteins were successfully depleted.

**Induction of RAGE Shedding via PKC—**Phorbol esters like PMA are known activators of PKC. To analyze whether PKC is involved in induced shedding of human RAGE, we treated RAGE-expressing cells with the PKC inhibitor Gö6976 and analyzed its influence on induced shedding of RAGE. The inhibitor Gö6976 is competitive with respect to the ATP binding site of PKC and selectively inhibits the calcium-dependent PKC isozymes PKCa (IC50 = 2.3 nM) and PKCβI (IC50 = 6.2 nM); Gö6976 does not affect the kinase activity of the Ca2+-independent PKC isoenzymes even at micromolar concentrations.

Cells were first pre-treated with Gö6976 (2 μM) for 1 h before the medium was replaced by medium supplemented either with each shedding inducer alone (PMA (1 μM) or the calcium ionophore Calcimycin (A23187, 2 μM), respectively) or with a shedding inducer in combination with the PKC inhibitor. After a 2-h secretion time, shedded RAGE was detected by Western blot analysis (Fig. 10A). Following pretreatment of cells with the PKC inhibitor Gö6976 the PMA and Calcimycin (A23187) induced shedding of RAGE was completely blocked when Gö6976 was present additionally to the shedding inducers during the time of secretion (Fig. 10B, bar 1 versus 3 and 5). This result shows that PKCa and/or PKCβI are involved in regulated ectodomain shedding of RAGE.

![Image 1](https://example.com/image1.png)

**FIGURE 6.** Proteolytic cleavage of RAGE at the cell surface. Cell-surface proteins of HEK-tgRAGE (A) and HEK-RAGE (B) cells were labeled with biotin. The amount of secreted (sRAGE) and cell-anchored biotinylated full-length RAGE (fl-RAGE) was determined separately after constitutive or stimulated RAGE shedding by Western blot analysis using antibody 9E10 (in A) or Mab5328 (in B). A, detection of isolated biotinylated RAGE variants at different time points of constitutive or PMA-induced shedding. Actin was detected in total cell lysates by Western blot analysis. B, shedding of biotinylated RAGE in the presence of either PMA (1 μM for 2 h) or APMA (0.14 mM for 12 min).

![Image 2](https://example.com/image2.png)

**FIGURE 7.** Localization of the RAGE cleavage site. **A**, scheme of investigated RAGE constructs: fl-RAGE, full-length RAGE, the black box (amino acids 343–363) represents the transmembrane anchor of RAGE, the C-terminus of RAGE is located in the cytosol; esRAGE, secreted recombinantly produced splice form of RAGE, because of a frameshift next to the codon for amino acid 331 the protein sequence differs from RAGE; sRAGEΔ, recombinant soluble form of RAGE ending after amino acid Ala-315. Recognition epitopes of the antibodies used are labeled at RAGE. B, Western blot analysis of proteins isolated from culture supernatant of RAGE-expressing HEK cells. sRAGE, sample from cells expressing full-length RAGE; esRAGE, sample from cells expressing the soluble splice variant esRAGE; sRAGEΔ, sample from cells expressing sRAGEΔ; mock, sample from untransfected cells. In the upper blot antibody Ab3260 was used for detection of RAGE, in the lower blot Mab5328 was used. C, partial alignment of the analyzed RAGE proteins. The epitope of Ab3260 is marked in gray, *, indicates that the protein sequence is normally continued at these positions.
DISCUSSION

In our report we demonstrate that the multiligand receptor RAGE is subjected to protein ectodomain shedding. By this event, the membrane-bound form of RAGE, which participates in a variety of pathophysiological conditions, is converted into a soluble counterpart. This observation is of particular interest, because several reports have assigned an antagonistic role of recombinant soluble RAGE in RAGE-mediated pathophysiological processes (24–26), and soluble RAGE-ligand complexes are thought to be eliminated from blood via the spleen and/or liver (27). So far generation of endogenous soluble RAGE forms has been limited to reports about alternative splice products (8, 11, 13–15, 28, 29).

After expression of full-length RAGE (55 kDa) in HEK cells we identified a 48-kDa soluble form of RAGE (sRAGE) and a 13-kDa cell-bound C-terminal fragment of RAGE (RAGE-CTF). The presence of sRAGE and RAGE-CTF as well as their sizes point to the proteolytic cleavage of full-length RAGE. We identified shedding of RAGE to be a constitutive process by which the ligand binding ectodomain of RAGE is released from the cell surface by a metalloproteinase-mediated proteolysis. Because shedding of RAGE could be inhibited by GM6001 (Galardin), a broad spectrum hydroxamic acid inhibitor, the RAGE sheddase(s) may belong to the large families of disintegrin metalloproteinases and matrix metalloproteinases (21). By applying the RNAi technology ubiquitously...
expressed metalloproteinases were targeted, and by this approach we were able to show that ADAM10 and MMP9 contribute to the shedding of RAGE. We also found that deficiency of ADAM17 and MMP2 had no influence on the shedding of RAGE in our cellular system. Consistent with our results, it was recently shown that fibroblasts isolated from ADAM10 null mice are largely devoid of RAGE shedding and fibroblasts of ADAM17 null mice are capable of shedding RAGE (30).

Several reports suggest that ADAM10 and ADAM17 are the main enzymes responsible for protein ectodomain shedding. A variety of substrates is cleaved by both ADAM10 and ADAM17, whereas several proteins are exclusively processed by either ADAM10 or ADAM17 (22). From our results we conclude that RAGE belongs to the class of substrates cleaved mainly by ADAM10. Besides ADAM10, we also identified MMP9 to be involved in RAGE shedding. MMP9 is normally an extracellular proteinase, which degrades basement membrane and extracellular matrix components. In addition, MMP9 was shown to be retained on the plasma membrane (31) and responsible for the ectodomain shedding of the transmembrane protein vascular epidermal growth factor (32) and intercellular adhesion molecule-1 (33). With the ability of membrane localization, MMP9 meets a requirement assigned to sheddases, and many non-matrix substrates have been identified as MMP substrates, including cell adhesion molecules, cell surface receptors, cytokines, and growth factors (23). Thus MMPs and ADAM proteins seem to be able to cleave similar families of proteins.

Because protein ectodomain shedding often proceeds subsequent γ-secretase cleavage, we analyzed the effect of γ-secretase inhibition on RAGE-CTF metabolism. After application of the γ-secretase inhibitor DAPT we detected an increase in the amount of RAGE-CTF generated by ectodomain shedding of RAGE. This observation suggests that RAGE-CTF is a substrate for γ-secretase. After ectodomain shedding the size of the remaining C-terminal stub likely determines whether a type I membrane protein becomes a substrate for subsequent γ-secretase processing: If the extracellular part of a C-terminal stub contains less than 50 amino acid residues, it is efficiently cleaved by γ-secretase (34). Using an antibody directed against the human RAGE epitope Val-316 to Gly-331, we were able to show that sRAGE released from full-length RAGE-expressing cells contains this epitope, and therefore human RAGE is cleaved about nine amino acids above the plasma membrane. Thus, RAGE-CTF meets the requirement for γ-secretase processing.

In the cellular system described here, shedding of RAGE was inducible via PMA-induced activation of Ca²⁺-dependent PKC isoenzymes PKCa and/or PKCβI. Interestingly, regulated protein ectodomain shedding often follows this route (20). After induction of RAGE shedding we found cellular levels of total full-length RAGE to be relatively unaltered.

Because total cellular RAGE includes RAGE located in the plasma membrane as well as RAGE present intracellularly in the endoplasmic reticulum, the Golgi apparatus, and vesicles, this observation suggests that only a subfraction of cellular RAGE is susceptible to proteolysis. By our biotinylation experiments we identified plasma membrane-located RAGE to be proteolytically cleaved. Similar results have been found for APP where shedding does not change the amount of total cellular APP (35), because cleavage of APP occurs predominantly on the cell surface and affects only a small pool of cellular total APP (36, 37). Nevertheless, proteolysis of APP plays a substantial role in the pathogenesis of Alzheimer disease.

The finding that RAGE is subjected to constitutive and regulated ectodomain shedding provides a comprehensible explanation for the observed discrepancy between different RAGE mRNA and protein levels. Despite the fact that in human lung ~80% of the RAGE mRNA encodes for full-length RAGE and only 7% for endogenous sRAGE (14) the full-length membrane-bound RAGE protein has been shown to be underrepresented as compared with the sRAGE protein (16). Proteolytic conversion of full-length RAGE into secreted RAGE, as shown in our study, is therefore obvious and may account to a large extent to generation of sRAGE in vivo.

In a recent study sRAGE and esRAGE levels were analyzed in association with different markers of cardiovascular risk in patients with type II diabetes. It was found that higher sRAGE levels were associated with higher esRAGE levels, but absolute sRAGE levels were ~4-fold higher (38). This is another indication that sRAGE and esRAGE are generated independently from each other.

Several lines of evidence suggest that RAGE plays important roles in some pathophysiological processes, including Alzheimer disease (AD). There is evidence that the amount of membrane-bound RAGE is increased in cerebral blood vessels in AD patients compared with control individuals (39), and in another study reduced levels of soluble RAGE were found in blood of AD patients (40). Thus, an increased amount of membrane-bound RAGE and the simultaneous decrease of sRAGE might be responsible for an enhanced Aβ uptake in sporadic AD cases.

By stimulating ectodomain shedding of RAGE the ratio between membrane-bound RAGE and its soluble counterpart should decline. This might result in a reduced uptake of Aβ into the brain and diminished RAGE-mediated neurotoxicity via cell membrane-bound RAGE. In this context, the results described here might form the basis for modulation of RAGE shedding by targeting distinct cellular signal transduction pathways involved in that process.

Addendum—During the course of the review process reports entitled “Calcium-regulated Intramembrane Proteolysis of the RAGE Receptor” (41) and “A Soluble Form of the Receptor for Advanced Glycation Endproducts (RAGE) Is Produced by Proteolytic Cleavage of the Membrane-bound Form by the Sheddase A Disintegrin and Metalloprotease 10 (ADAM10)” (30) were published.
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