Biochemical and Biophysical Research Communications 370 (2008) 1–5

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Calcium-regulated intramembrane proteolysis of the RAGE receptor

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ARTICLE INFO

Article history:
Received 25 February 2008
Available online 18 March 2008

Keywords:
RAGE
Receptor
Ectodomain shedding
Calcium
RIP
ADAM
γ-Secretase

ABSTRACT

The receptor for advanced glycation endproducts (RAGE) interacts with several ligands and is involved in various human diseases. RAGE_v1 or sRAGE, a RAGE splice variant, is secreted and contributes to the removal of RAGE ligands. Because RAGE blockade by specific antibodies directed against RAGE extracellular domains and the use of sRAGE have been proven to be beneficial in the context of pathological settings, both RAGE and sRAGE are considered as therapeutic target. Here, we show that sRAGE is also produced through regulated intramembrane proteolysis of the RAGE receptor, which is catalyzed by ADAM10 and the γ-secretase and that calcium is an essential regulator of RAGE processing. Furthermore, RAGE intracellular domain localizes both in the cytoplasm and the nucleus and induces apoptosis when expressed in cells. These findings reveal new aspects of RAGE regulation and signaling and also provide a new interaction between RAGE and human pathologies.

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The receptor for advanced glycation endproducts (RAGE), a member of the immunoglobulin superfamily of cell membrane receptors [1], interacts with various ligands such as members of the S100 protein family, amyloid-beta (Aβ), advanced glycation endproducts (AGEs) or amphoterin (HMGB1) [1–6] and is involved in cancer, inflammation, diabetes and Alzheimer disease [5–12]. RAGE is a type I transmembrane receptor composed of three immunoglobulin domains located in the extracellular space, a single membrane spanning domain and a short cytoplasmic region. Several splicing forms of the *RAGE* gene have been characterized [13], and, among them, RAGE_v1 or sRAGE, lacking both the membrane and intracellular domains, is secreted in the extracellular space and acts as a decoy for RAGE ligands inhibiting their interaction with RAGE present at the cellular membrane and subsequently preventing RAGE overactivation [9,14,15]. The use of sRAGE in mouse models has been shown to be beneficial in pathological settings and there is also a strong correlation between sRAGE levels in the human serum and various pathologies [16]. However, the two immunological assays used to monitor sRAGE levels (detecting total circulating sRAGE versus an assay specific for RAGE_v1 splice variant) [13] revealed some differences which suggest that other mechanisms may be responsible for sRAGE production. Furthermore, some proteins and receptors presenting a similar topology as RAGE (Notch, APP, ErbB-4 or LRP) undergo constitutive or induced regulated intramembrane proteolysis (RIP) catalysed by ADAM metalloproteinases and the γ-secretase [17]. Here, we show that RAGE is also a substrate of RIP which leads to sRAGE and RAGE intracellular domain (RICD) release in the extracellular space and the cytoplasm/nucleus, respectively, and that calcium (Ca^{2+}) is an essential regulator of RAGE processing. Furthermore, expression of RICD in cells promotes apoptosis.

Materials and methods

Generation of RAGE intracellular domain antibody. RAGE intracellular domain (amino-acids 361–404 of the human RAGE protein) was cloned in frame with GST in the pGEX vector and the fusion protein was expressed in *Escherichia coli*. RAGE intracellular domain protein was then cleaved from GST by the factor Xa protease and purified. The purity of the domain was assessed by mass-spectrometry and amino-acid analysis. Purified RAGE intracellular domain was used to produce the rabbit RAGE C-term antibody. The produced antibody only recognizes RAGE intracellular domain and was used in this study at a dilution of 1/1000 for Western blotting (WB) and 1/500 for cytoimmunochemistry.

HEK293 cells treatment. Control and RAGE stable HEK293 cells lines (Weibel M., Gemperle C., Heizmann C.W. and Galichet A., submitted) were plated in DMEM medium containing 10% fetal bovine serum, 2 mM glutamine and streptomycin/penicillin/G418 antibiotics. After plating, cells were cultured for 24 h and were then treated with ionomycin, PMA, thapsigargin, RAGE ligands in serum-free medium for the indicated time before analysis. Expression of human ADAM10-HA in control and RAGE HEK293 cells was achieved by transfection with lipofectamine (invitrogen) following manufacturer's instructions for 16 h. Medium was then replaced with serum-free medium for 10 h before analysis.

Lung tissue preparation. Fifteen-months-old wild-type C57BL/6 and $RAGE^{-/-}$ [18] mice were anesthetized and perfused with PBS. Lungs were then removed and were homogenized in 50 mM Tris–HCl pH8, 2% SDS and 1% Triton X-100 in presence of protease inhibitors (Roche). Cellular lysates were centrifuged at 100,000*g* and at 8 °C for 1 h. Supernatants were used for Western blotting analysis.

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doi:10.1016/j.bbrc.2008.02.163

Primary cortical neurons culture and treatment. Rat cortical neurons were prepared and maintained as described [19]. They were treated after 8 days in culture with 2 μM ionomycin or thapsigargin for 20 min before analysis by Western blotting and immunocytochemistry.

Cloning and expression of RICD-HA. Human RAGE intracellular domain (sequence corresponding to amino-acids 361–404) was cloned in frame with the HA-tag in the pUK-BK-C vector and the construct was sequenced. The construct was transiently expressed in HEK293 cells using Lipofectamine2000 (Invitrogen), according to the manufacturer's instructions for 16 h.

Western blotting, immunofluorescence, and antibodies. Human RAGE Vdomain, β-tubulin, phosphorylated p38, p38, phosphorylated JNK and JNK antibodies, procedures for protein extraction from cells, Western blotting, cytoimmunochemistry as well as cell viability and TUNEL assays were already described [20]. Statistical significance was determined using ANOVA single factor and Student's *t*-test ($^{***}p = 0.0001$).

Results and discussion

First, we examined whether RAGE could be a substrate of RIP. For this purpose, HEK293 RAGE cells (Weibel M., Gemperle C., Heizmann C.W., and Galichet A., submitted) were treated with two inducers of the ADAM proteins, phorbol 12-myristate 13-acetate (PMA) and the Ca^{2+}-ionophore ionomycin [21]. Whereas PMA did not induce RAGE cleavage, ionomycin treatment leaded to formation of RAGE ectodomain (sRAGE) in the culture medium and C-terminal domain (C-term RAGE) in the cells (Fig. 1A). Moreover, RAGE RIP was also observed constitutively (Fig. 1A) and was increased by cell density (data not shown). In contrast, RAGE ligands (Aβ, AGEs, HMGB1 and S100 proteins) did not induce RAGE processing neither after 20 min nor after 2 h (Fig. 1B and data not shown). As ionomycin preferentially induces ADAM10, we then transiently transfected control and RAGE cells with ADAM10 and could observe increased RAGE processing as well as sRAGE accumulation in the extracellular medium (Fig. 1C), suggesting that ADAM10 is likely to be the metalloproteinase involved in RAGE RIP.

Ca^{2+}, as intracellular signal molecule, regulates many cellular functions and processes [22,23]. The influence of ionomycin on RAGE shedding indicated that changes in Ca^{2+} concentration could affect RAGE processing. Indeed, addition of the Ca^{2+} chelator EGTA in the culture medium prevented constitutive and ionomycin-in-

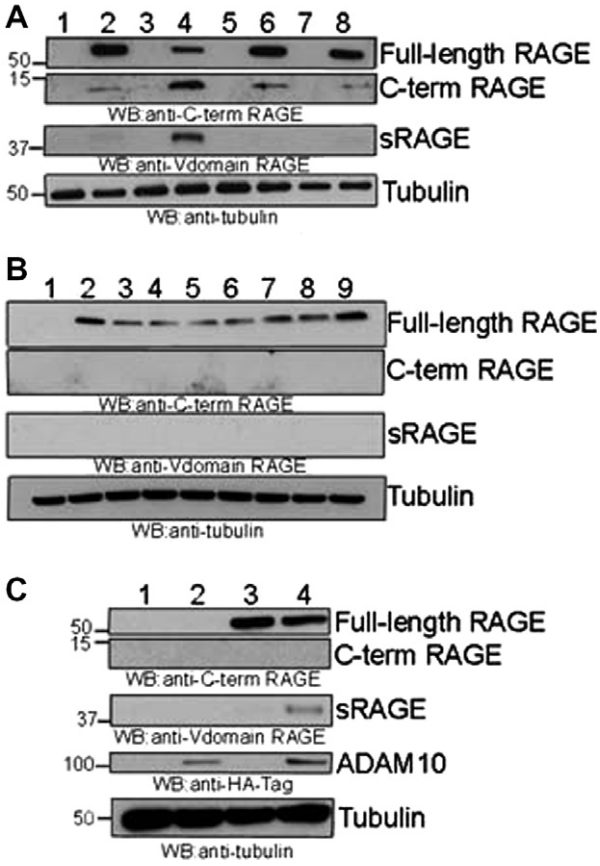

Fig. 1. Human RAGE undergoes inducible and constitutive regulated intramembrane proteolysis in HEK293 cells. (A) Control (1, 3, 5 and 7) and RAGE (2, 4, 6 and 8) cells were treated with DMSO (1, 2, 5 and 6), 1 μM ionomycin (3 and 4) or 1 μM PMA (7 and 8) for 10 min. Cell lysates were immunoblotted with the C-term RAGE antibody and tubulin was used as loading control. Conditioned media were concentrated and were immunoblotted with the Vdomain RAGE antibody. All protein sizes are given in kDa. (B) RAGE cells were treated with 1 μM Aβ oligomers (3), Aβ fibrils (4), Aβ aggregates (5), S100B (6) and S100A1 (7) dimers, HMGB1 (8) or AGE albumin (9) for 20 min. Cell lysates and conditioned culture media were immunoblotted as indicated. (C) Control (1 and 2) and RAGE (3 and 4) cells were mock (1 and 3) or transfected with the human ADAM10 (2 and 4) for 16 h. Lysates and culture media were immunoblotted as indicated.

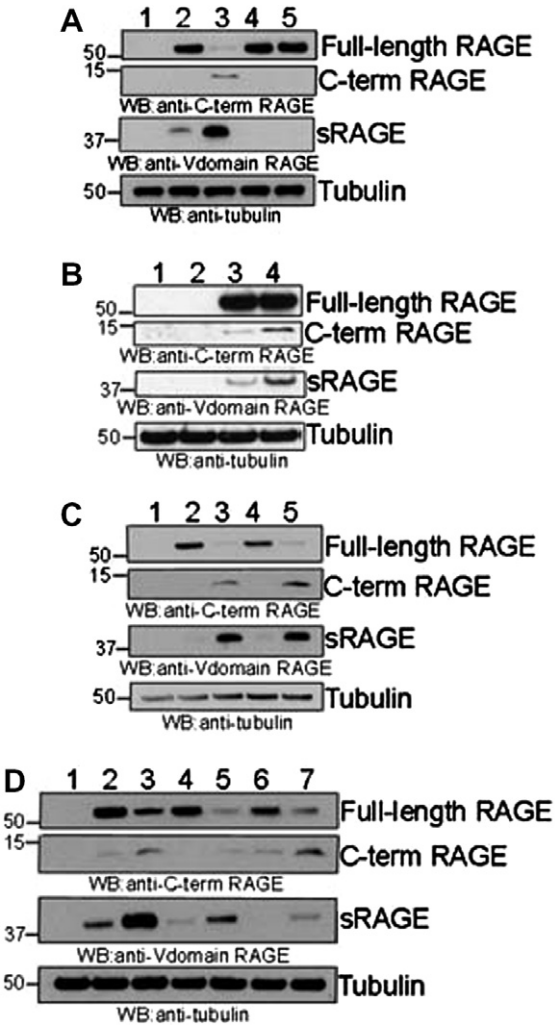

Fig. 2. Role of calcium in RAGE processing. (A) Control (1) and RAGE (2 to 5) cells were pre-treated with water (2 and 3) or 2 mM EGTA (4 and 5) for 10 min. RAGE cells were treated with DMSO (2 and 4) or 1 μM ionomycin (3 and 5) for 10 min. Cell lysates and conditioned media were immunoblotted as indicated. All protein sizes are given in kDa. (B) Control (1 and 2) and RAGE (3 and 4) cells were treated with DMSO (1 and 3) or 1 μM thapsigargin (3 and 4) for 10 min. Cell lysates and conditioned media were immunoblotted as indicated. (C) Control (1) and RAGE (2 to 5) cells were pre-treated with DMSO (2 and 3) or 1 μM DAPT (4 and 5) for 24 h. RAGE cells were then treated with DMSO (2 and 4) or 1 μM ionomycin (3 and 5) for 10 min. Lysates and conditioned media were immunoblotted as indicated. (D) Control (1) and RAGE (2 to 7) cells were pre-treated with DMSO (1 to 3), 10 μM clasto-lactacystin β-lactone (4 and 5) or 10 μM MG-132 (6 and 7) for 24 h. RAGE cells were then treated with DMSO (2, 4 and 6) or 1 μM ionomycin (3, 5 and 7) for 10 min. Lysates and conditioned media were immunoblotted as indicated.

duced RAGE processing (Fig. 2A). Furthermore, thapsigargin, a specific sarco-endoplasmic reticulum Ca^{2+}-ATPase inhibitor, which liberates Ca^{2+} from the endoplasmic reticulum and leads to its accumulation in the cytoplasm, also induced sRAGE and C-term RAGE accumulation (Fig. 2B), demonstrating that modulation of Ca^{2+}-concentration is an essential regulator of RAGE processing.

Antibody directed against RAGE cytoplasmic sequence (RAGE C-term antibody) detected a single peptide of an apparent molecular mass of about 12 kDa (designated as C-term RAGE). To investigate whether this peptide was the substrate of the γ-secretase and, consequently, contained the RAGE transmembrane and cytoplasmic domains, or the product resulting from this cleavage which would only contain RAGE cytoplasmic domain, RAGE cells were treated with DAPT, a γ-secretase inhibitor [24]. DAPT treatment resulted in enhanced accumulation of C-term RAGE in presence of ionomycin, suggesting that C-term RAGE is the substrate of the γ-secretase (Fig. 2C). In addition, the elevation of C-term RAGE levels could not result from increased RAGE shedding, because sRAGE secretion was not affected by DAPT treatment. As a result of γ-secretase cleavage, C-term RAGE should be converted to the RAGE intracellular domain (RAGE ICD, RICD). However, RICD generation could neither be observed after constitutive nor following ionomycin-induced RAGE RIP. Similarly to what is known for APP or Nectin 1 [25,26], RICD might be quickly degraded after its generation. Several reports suggest a potential role of the proteasome for degradation of released ICD [17]. RAGE cells were treated with or without ionomycin in the presence of the specific proteasome inhibitor clasto-lactacystin β-lactone or of the peptide aldehyde proteasome inhibitor MG-132. Nevertheless, none of them revealed the presence of RICD, suggesting that the proteasome might not be involved in RICD degradation (Fig. 2D). MG-132 is also a weak γ-secretase inhibitor [27], and, similarly to DAPT, it also increased C-term RAGE accumulation, which further confirmed the involvement of γ-secretase in RAGE processing.

The constitutive and inducible RAGE RIP observed in HEK293 cells prompted us to investigate whether this process also occurs *in vivo*. Western blot analysis of mouse lung homogenates revealed the presence of bands with an apparent molecular mass of about 55 (Full-length RAGE) and about 12 kDa (RAGE C-term fragment) with the RAGE C-term antibody and about 55 (Full-length RAGE) and about 45 kDa (sRAGE) with RAGE Vdomain antibody, and none of those bands were detected in RAGE knock-out lung extract, suggesting that RAGE processing occurs also *in vivo* (Fig. 3A). To confirm the physiological influence of modulation of Ca^{2+} concentration on RAGE processing, rat cortical primary neurons, expressing RAGE, were treated with ionomycin or thapsigargin, and, in this model, thapsigargin was more potent to induce the formation of RAGE C-term. But, similarly to the situation observed in RAGE HEK293 cells, RICD could not be detected by Western blot (Fig. 3B). Immunocytochemistry was then performed to reveal the presence of RICD and to observe changes in subcellular localization that could occur following induction of RAGE processing by thapsigargin. Antibody directed against the extracellular part of RAGE showed a punctuated staining in untreated cells (Fig. 3C) which was decreased in presence of thapsigargin, suggesting that the amount of RAGE present at the membrane was decreased following treatment with thapsigargin (Fig. 3C). RAGE C-term antibody revealed a faint signal in the nucleus of untreated cells, indicating that, similarly to what was observed in HEK293 cells, there was a constitutive turn-over of RAGE in cortical neurons (Fig. 3C). This observed nuclear staining was then increased following the addition of thapsigargin (Fig. 3C). These observations suggest that RAGE RIP results in the formation of RICD which translocates to the nucleus.

RAGE processing occurs both *in vitro* and *in vivo*, is induced by modulations of Ca^{2+}-concentrations, and results in the formation

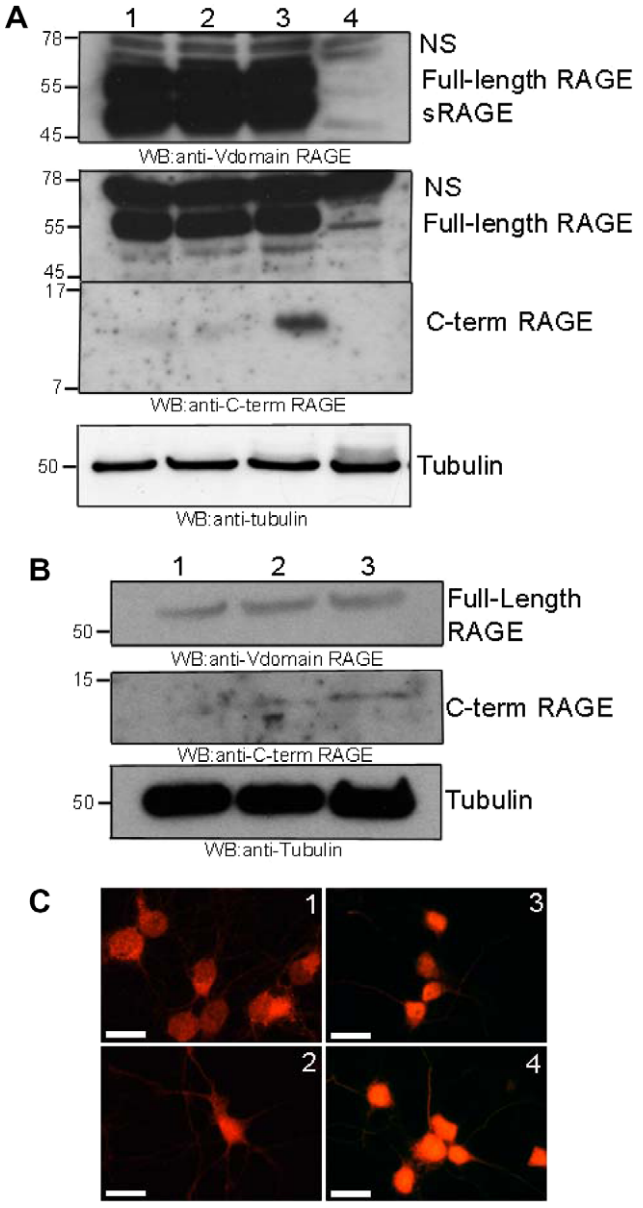

Fig. 3. RAGE processing *in vivo*. (A) Total proteins from 15-months-old wild-type and $RAGE^{-/-}$ lung tissues were extracted in presence of 1% triton and 2% SDS and lysates were immunoblotted as indicated. Protein sizes are given in kDa and N.S. indicates non specific bands revealed by the antibody. (B) Primary rat cortical neurons (DIV8) were treated with DMSO, 2 μM ionomycin or 2 μM thapsigargin for 20 min. Cell lysates were immunoblotted as indicated. (C) Rat cortical neurons were treated with DMSO or 2 μM thapsigargin for 20 min and cells were labeled with antibodies as indicated. Scale bar, 5 μm.

of sRAGE, released in the extracellular medium, and RICD which translocates to the nucleus. The ICDs, produced by cleavage of Notch, APP or ErbB-4, modulate transcriptional activity, promote cell differentiation or induce apoptosis in physiological and pathological processes [17,28,29]. It raised the question whether RICD formation could also affect cellular functions. As RICD seems to be quickly degraded after its formation, we transiently expressed RAGE intracellular domain fused to a HA-tag in HEK293 cells (Fig. 4A). RICD-HA fusion protein was detected both in the cytoplasm and in the nucleus of transfected cells (Fig. 4B). In addition, some cells also showed a strong staining in the nucleus only and presented morphological changes such as formation of condensed nuclei and cell shrinkage (Fig. 4C). Because these alterations are hallmarks of cellular apoptosis [30], we analyzed the effect of

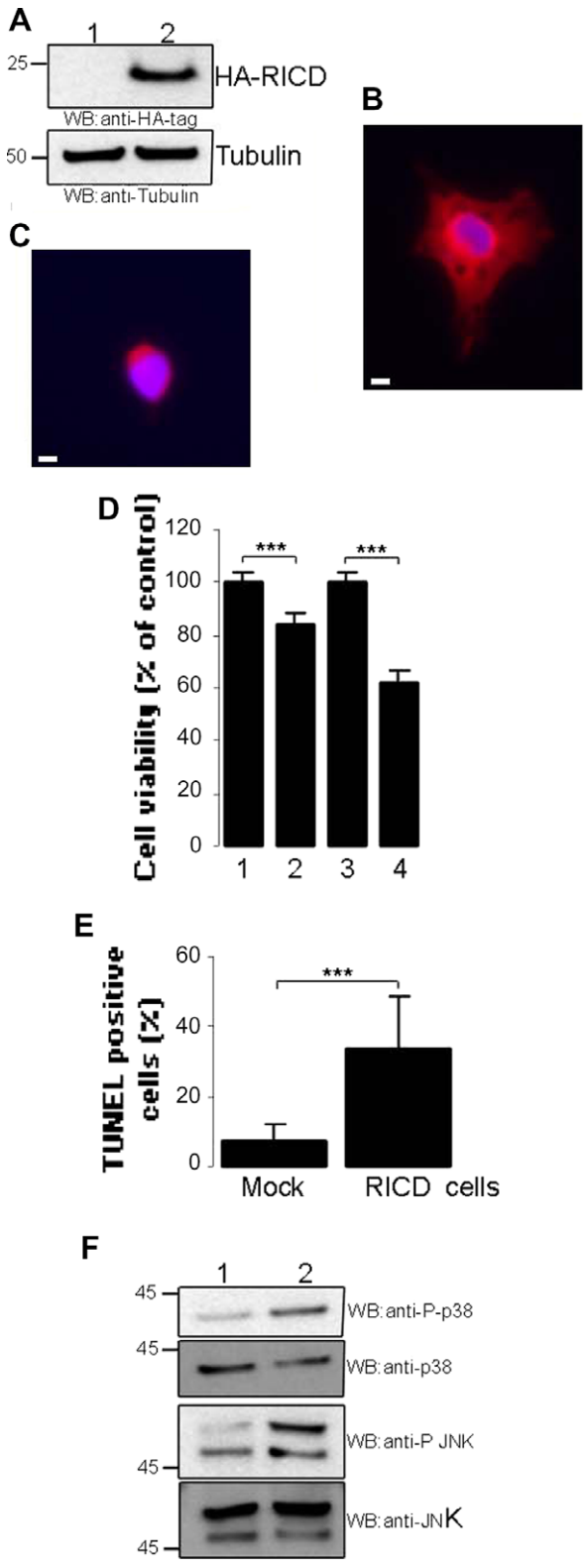

Fig. 4. RAGE intracellular domain induces apoptosis in HEK293 cells. (A) Lysates of mock (1) and RICD-HA (2) transfected HEK293 cells were immunoblotted as indicated. All protein sizes are given in kDa. (B and C) RICD-HA HEK293 cells were labeled with an anti-HA antibody (red) and DAPI (blue). Scale bar, 5 μm. (D) MTT assay on mock (1 and 3) and RICD-HA (2 and 4) transfected cells after 16 (1 and 2) and 40 h (3 and 4) (means ± standard deviations, $n = 16$, $^{***}p = 0.0001$). (E) Percentage of TUNEL positive cells in mock and RICD-HA transfected cells (means ± standard deviations, $n = 2$, $^{***}p = 0.0001$). (F) Lysates of mock (1) and RICD-HA (2) transfected cells were immunoblotted as indicated.

RICD-HA protein on cell viability and found 16% and 38% decrease in cell viability 16 and 40 h after transfection, respectively (Fig. 4D). The induction of apoptosis in presence of RICD was also confirmed by the increase of TUNEL positive cells 16 hours after transfection (Fig. 4E). In addition, expression of RICD also induced the activation of the p38 and JNK MAP kinases (Fig. 4F) which are stress-related kinases involved in the induction of apoptosis [31].

Collectively, our data demonstrate that RAGE is processed by subsequent cleavage mediated by the ADAM10 metalloprotease and the γ-secretase and that this process results in the release of sRAGE and RICD in the extracellular space and in the cytoplasm/nucleus, respectively. In addition, it also demonstrates a novel functional interaction between RAGE receptor and intracellular signaling which is of particular importance in the context of RAGE functions in human pathologies and opens new perspectives in using RAGE as therapeutic target.

Acknowledgments

The authors thank U. Konietzko for providing the DAPT inhibitor, ADAM10 construct and the pUK-BK-C vector and for valuable discussions as well as I. Vodopivec and S. Cattepoel for their help with rat cortical neurons. We also thank A. Bierhaus for the RAGE$^{-/-}$ mice. This work was supported by the NCCR "Neural Plasticity and Repair" to C.W.H. and by a grant from the Foundation for Research at the Medical Faculty, University of Zurich to A.G.

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