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Calcium-regulated intramembrane proteolysis of the RAGE receptor

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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.

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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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-

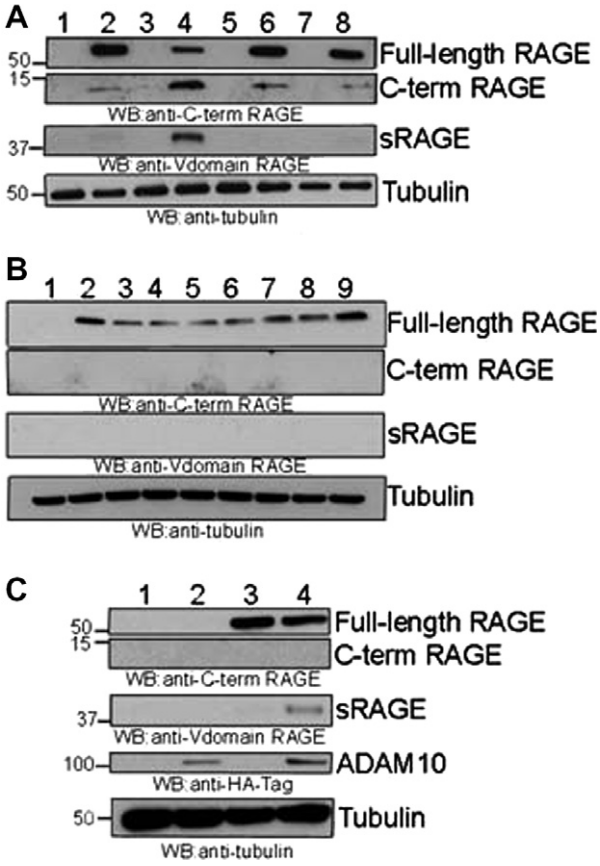

Fig. 1. Human RAGE undergoes inducible and constitutive regulated intramembrane proteolysis in HEK293 cells. (A) Control (1, 3, 5 and 7) and RAGE (2, 4, 6 and 8) cells were treated with DMSO (1, 2, 5 and 6), 1 μM ionomycin (3 and 4) or 1 μM PMA (7 and 8) for 10 min. Cell lysates were immunoblotted with 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.

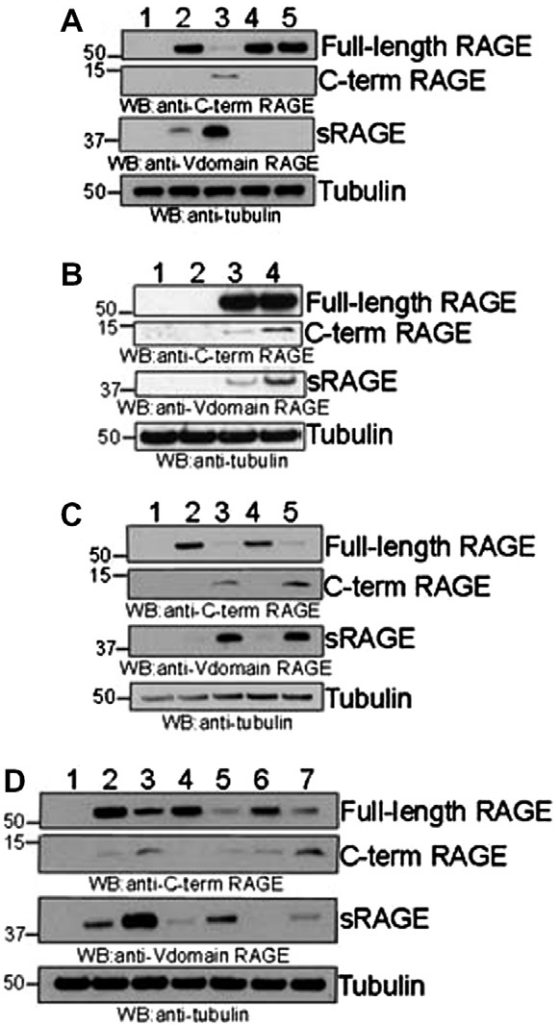

Fig. 2. Role of calcium in RAGE processing. (A) Control (1) and RAGE (2 to 5) cells were pre-treated with water (2 and 3) or 2 mM EGTA (4 and 5) for 10 min. RAGE cells were treated with DMSO (2 and 4) or 1 μM ionomycin (3 and 5) for 10 min. Cell lysates and conditioned media were immunoblotted as indicated. 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.