Structural insights into the oligomerization mode of the human Receptor for Advanced Glycation End-products (RAGE)

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Running title

RAGE can homodimerize through different modules

Abbreviations

AGE: Advanced Glycation End-Product; EGF: Epidermal Growth Factor; ICAM-1: Intercellular Adhesion Molecule 1; GpA: Glycophorin A; Ig: Immunoglobulin; MR: Molecular Replacement; RAGE: Receptor for Advanced Glycation End-products; SAXS: Small-Angle X-ray Scattering; TM: transmembrane.

Keywords

RAGE; receptor oligomerization; structure; signal transduction; adhesion molecule

Abstract

RAGE is a pattern recognition receptor sensing endogenous stress signals associated with the development of various diseases including diabetes, vascular complications, Alzheimer's disease and cancer. RAGE ligands include AGEs, S100 proteins, HMGB1 and amyloid β -peptides/fibrils. Their signaling through RAGE induces a sustained inflammation that accentuates tissue damage, thereby participating in the disease progression. Receptor oligomerization seems to be a crucial parameter for the formation of active signaling complexes although the precise mode of oligomerization remains unclear in the context of these various ligands. Here, we present the first crystal structure of the VC1C2 fragment of the RAGE ectodomain. This structure provides the first description of the C2 domain in the context of the entire ectodomain and supports the observation of its conformational freedom relative to the rigid VC1 domain tandem. In addition, we have obtained a new crystal structure of the RAGE VC1 fragment. The packing in both crystal structures reveals an association of the RAGE molecules through contacts between two V domains and the physiological relevance of this homodimerization mode is discussed. Based on homology with single-pass TM receptors, we also suggest RAGE dimerization through a conserved GxxxG motif within its transmembrane domain. A multimodal homodimerization strategy of RAGE is proposed to form the structural basis for ligand-specific complex formation and signaling functions as well as for RAGE-mediated cell adhesion.

Introduction

The Receptor for Advanced Glycation End-Products (RAGE) is a cell surface receptor from the Immunoglobulin (Ig) superfamily [1]. This 50-55 kDa glycosylated protein is expressed in various cell types during development but its expression is severely repressed in mature animals, except in certain pulmonary and neuronal cells [2-3]. RAGE consists of an extracellular region bearing three Ig domains belonging respectively to the V-, C-, and S-type [4] (V, C1, and C2 domains), a single transmembrane (TM) spanning helix, and a short cytoplasmic tail devoid of kinase activity and presumably unstructured [1]. A wide variety of ligands binding to the RAGE ectodomain have been identified including Advanced Glycation End-products (AGEs), S100 proteins, High Mobility Group Box 1 protein (HMGB1), amyloid β -peptides and fibrils, nucleic acids, phospholipids and glycosaminoglycans [1,5-12]. Recently, RAGE was shown to synergize with several components of the complement system to stimulate an innate immune response, such as the anaphylatoxin C3a [13], component C1q [14], and complement receptor 3 (also termed $\alpha_M\beta_2$ integrin or Mac-1) [15]. These latter interactions are believed to promote leukocyte recruitment at inflammatory sites [15] and phagocytosis [14].

Despite their divergence in terms of molecular architecture, tissue localization, and cellular function under homeostatic conditions, a hallmark of many RAGE ligands is their tendency to accumulate at inflammatory sites during the pathogenesis of various diseases [16-18], thereby acting as damage-associated molecules. RAGE is therefore a pattern recognition receptor sensing endogenous stress signals [19], and ligand recognition by its ectodomain triggers successively signal transduction, adaptor protein recruitment by the receptor intracellular tail [20-21] and activation of downstream signaling cascades, in a cell-specific manner [22-23]. In most cases, this leads to transcription factor activation (NF κ B, STAT3,

and others) and to the subsequent production of pro-inflammatory molecules. During this process, gene expression of the receptor itself is often upregulated, thereby feeding an amplification loop propagating the cellular dysfunction through extensive cell activation [22-23]. Thus RAGE-sustained inflammation imposes a massive stress on the cells, which leads to further tissue damage rather than healing and recovery [19,22-23].

RAGE blockade has been considered as a promising strategy in the treatment of cancers, diabetic complications and neurodegenerative disorders [24-26]. The task of designing RAGE inhibitors for therapeutical use is however complicated by the diversity of its ligands and the fact that RAGE-mediated signaling can also have a protective effect in tissue homeostasis and resolution of inflammation [23]. Thus, a prerequisite for efficient targeting of RAGE is the knowledge of the intimate mechanisms that govern ligand recognition and signal transduction by the receptor. Structural models are available for isolated fragments of the receptor ectodomain [27-29], as well as for a short segment of the RAGE intracellular tail [30]. As for many receptors bearing a single TM span, it is believed that ligand binding and subsequent receptor activation leading to signal transduction require RAGE oligomerization. RAGE has been proposed to exist as preformed, unstable oligomers on the cell surface [31]. RAGE homodimerization through V-V domain contacts is observed in solution [32] and self-association is enhanced by S100B and AGEs binding both *in vitro* and in cells [33]. S100A12 binds to the receptor via a dimeric C1-C1 interface [34], and part of the RAGE molecules present at the cell surface may associate through a C2-C2 disulfide crosslinked dimer [35]. RAGE has recently been shown to form stable hexamers in the presence of heparan sulfate and hexamerization of the receptor seemed to be essential for the formation of active signaling complexes with several ligands [36]. However, the mechanism leading to signal transduction across the plasma membrane upon ligand binding is still unclear.

To address this question in structural details, we have undertaken crystallographic studies of the human RAGE ectodomain. Here we report the crystal structure of the full-length human RAGE ectodomain (VC1C2 module) and that of the RAGE VC1 module obtained in a new crystal form as compared to previously published structures [28-29], at 3.8 and 2.4 Å resolution, respectively. The presence of similar crystal contacts in both our structures and in other structures of human and mouse RAGE VC1 fragments led us to propose that RAGE V-V homodimerization may constitute one mode of self-association for the receptor. We also suggest that RAGE might self-associate through its transmembrane segment. Based on these models and other dimerization modes reported in the literature, we propose that RAGE uses a multimodal homodimerization strategy that allows formation of ligand-specific complexes that are relevant not only for signaling functions but also for RAGE-mediated cell adhesion.

Results

To gain insights into how self-association of the RAGE receptor occurs, we crystallized the VC1 fragment (residues Ala23 to Glu231) and the VC1C2 fragment (residues Ala23 to Pro323) of the human receptor (hRAGE). We obtained a new crystal form for the VC1 fragment, which diffracted X-rays to 2.4 Å resolution (Table 1). Crystals of hRAGE VC1C2 diffracting to 3.8 Å resolution and belonging to space group P6₅ were also obtained (Table I). In contrast to various monomeric VC1 search models that failed to give a molecular replacement (MR) solution, a crystal packing dimer observed in the VC1 structure presented in this paper was successful as a MR search model (Z-score>20) and the electron density maps obtained after initial refinement confirmed the association of the two VC1C2 molecules contained in the asymmetric unit through their respective V domains. A well-defined

additional density was also visible for one of the two missing C2 domains (Fig. S1A-B), for which a model could be placed manually based on the unpublished NMR structure (RCSB entry **2ENS**). No electron density was visible for the second C2 domain which could therefore not be modeled (Fig. S1C-D).

The structure of the full-length human RAGE ectodomain (VC1C2 fragment) is depicted in Fig. 1. The VC1 part of this model is very similar to the structures that have been previously published [28-29,36] as well as to the VC1 structure described in this paper (Fig. S2). As reported before, the V domain adopts an atypical V-type Ig fold with a very short C' strand and no C'' strand, while the C1 domain folds into a classical C-type Ig domain. For the VC1 part, regions of divergence between all reported structures are mostly restricted to flexible loops bridging the various β -strands (Fig. S2). The most pronounced rearrangements are observed for the loop connecting strands C' and D in the V domain (residues 64 to 75) where a short 3_{10} helix is observed in the structures reported here as well as in the MBP-VC1 structure [29]. The orientation between the V and C1 domains is conserved in all structures, in agreement with the idea that VC1 acts as a rigid, structural unit within the receptor ectodomain [28,37].

The data presented here also provide the first crystallographic structure of the third Ig module of RAGE ectodomain and is in good agreement with the structure of the C2 domain determined by NMR (Fig. 1C-D). The r.m.s.d. on C_{α} atoms between the C2 domain of the VC1C2 structure and the best representative model from the NMR structure is 1.85 Å. As expected, the C2 domain (residues 238 to 321) adopts an Ig fold that belongs to the S-type [4], with however small discrepancies in comparison with the canonical topology (Fig. 1C-D). The first strand of the domain is split into two parts: the first half (strand A) associates with the first β -sheet while the second half (strand A') packs against the second β -sheet. The last strand appears to be divided into two parts as well (G and G') both held by main chain

hydrogen bonds within the second β -sheet. Finally, there is no strand C' in the C2 domain structure: the corresponding region, which is highly rich in proline residues, rather forms an unstructured loop. Due to the low resolution of the diffraction data, the secondary structure assignments for the C2 domain should be considered as putative. As for the other two Ig domains of RAGE, the C2 domain is further stabilized by an internal disulfide bridge linking Cys259 in strand B and Cys301 in strand F (Fig. 1D).

In the structure of hRAGE VC1C2, the linker connecting the C1 and C2 domains (residues 231 to 237) is quite extended and there is no interaction between C2 and the two N-terminal RAGE domains (Fig. 1A). The molecule therefore adopts a quite elongated shape similar to the one observed in solution [32]. As suggested previously, the C1-C2 linker also confers a high degree of mobility to the C2 domain relatively to the VC1 rigid block [37] and act as a pendular lever to allow free rotation of the C2 domain around the VC1 module.

The hRAGE VC1 and hRAGE VC1C2 structures described in this paper overall display very comparable crystal contacts between two V domains, i.e. an antiparallel, elongated dimer paired through the V domains of each monomer (Fig. 2 & 3). A very similar crystal packing involving V-V domain contacts has also been observed in the structure of hRAGE VC1 obtained in a P2₁2₁2 crystal symmetry [28] although the authors pointed out a different type of dimer as being physiologically relevant for RAGE function (Fig. S4B). Recently, the crystal structure of mouse RAGE VC1 in complex with heparan sulfate (RCSB entry **4IM8**) also revealed similar V-V domain contacts within the crystal [36], suggesting that it is not specific to hRAGE (Fig. S4C). In both hRAGE VC1 and VC1C2 structures reported here, the V-V packing dimer interface is formed by the first β -sheet of each monomer (strands BDE) as well as the loop region and the 3_{10} helix. Analysis of the more accurate VC1 structure with PISA [38] revealed that the interaction between the two VC1 modules buries almost 1100 Å². The primary interaction that bridges the two V domains is a

π - π stacking between the aromatic rings of Phe85 from the two symmetry-related V domains (Fig. 2B). This is the sole, direct interaction between two residues at the dimer interface and it takes place within a highly conserved hydrophobic pocket encompassing residues Pro33, Val35, Leu79, Pro80, and Pro87 from each monomer. Phe85 is either conserved or replaced by a Leu residue in RAGE sequences from other organisms, suggesting that a similar type of interaction between RAGE V domains could occur in many different species.

Outside the hydrophobic core, a network of water molecules further stabilizes the peripheral regions of the intermolecular interface. Water-mediated interactions are formed between the side chains of Lys37, Asn81 and Ser83 on one V domain, and the side chain of Glu32 as well as the carbonyl groups of Gly31 and Pro87 main chains on the other V domain. Additionally, Lys43 side chain from each monomer is connected, through water molecules, to the side chains of Arg29 and Gln119, as well as the carbonyl groups of Ile120 and Gly148 main chains from the other monomer (Fig. 2B). These latter polar interactions allow to partially fix the respective orientation of the two VC1 molecules by bridging the V domain of one monomer to the C1 domain of the second monomer. However, since these interactions are mostly dependent on the surrounding hydration shell, a certain degree of flexibility exists between the two monomers. Indeed, superimposition of the VC1 dimer moiety from both VC1 and VC1C2 crystals reveals that the respective orientation of the two V domains is not identical. The different orientation between the two structures can be described by a rotation perpendicular to the dimer 2-fold axis that mostly affects the bottom part of the V-V interface where the two symmetrical C'-D loop regions are either closer or further away from each other (Fig. 2C-D). The C-terminal end of the C1 domain is thereby shifted by 11 Å between the two structures (Fig. 2C). This variance in the crystal contacts indicates that the interaction is rather weak and that the interface between the V domains is slightly flexible.

In solution, an equilibrium between monomeric and V-V homodimeric recombinant RAGE already exists in the absence of ligands [32,39]. In particular, deuterium exchange experiments performed on the full-length RAGE ectodomain free of ligands revealed that β -sheet BDE has the lowest proton exchange rate [39], in good agreement with the formation of the V-V dimer interface suggested by crystal packing. This surface is buried at the V-V interface but would be totally exposed to the solvent in monomeric RAGE. It is however still of debate whether such a V-V homodimer exists *in vivo*. Zong *et al.* [33] have shown that a recombinant RAGE V domain is able to block RAGE self-association in HEK293T cells. In addition, mutations of residues in the equivalent dimer interface of mouse RAGE inhibited dimer formation and impaired further hexamerization in the presence of heparin-derived oligosaccharides [36]. Finally, a monoclonal antibody presumably targeting residues in or around this dimer interface of mouse RAGE interfered with RAGE signaling and downstream ERK_{1/2} phosphorylation [36]. All together, these data suggest that the RAGE V domain plays a role in mediating the receptor homodimerization *in vivo*. However it remains uncertain whether it would be through V-V contacts or through V domain interaction with other regions of the RAGE ectodomain.

To date, all available structural data describing a dimerization through the V domain have been obtained with a protein expressed recombinantly in bacteria [28,36, this study]. Glycosylations on the endogenous receptor might interfere with the formation of the V domain dimer *in vivo* since two glycosylations sites have been identified in RAGE V domain at positions 25 and 81 [40-41]. Asn25 is located at the beginning of strand A and is therefore totally exposed to the solvent both in a monomeric and a V-V homodimeric RAGE model. Asn81 is situated in the D-E loop, at the upper part of the putative dimer interface. However, its side chain is pointing towards the solvent. To analyze whether V-V homodimerization would still be possible with a fully glycosylated receptor, we constructed a model for RAGE

glycosylated on Asn81 (Fig. S5) using Glyprot [42]. We used a glycan chain encompassing seven carbohydrate moieties (two N-acetyl-D-glucosamine groups followed by five mannose groups branched in two antennas – see Fig. S5A) which corresponds to the complex-type glycosylation pattern identified for murine RAGE [41]. In the resulting model, the glycan chain of Asn81 from one monomer makes only few clashes with the second monomer (Fig. S5B) and these clashes are mostly restricted to contacts with the side chains of charged residues. Thus, a simple rotation of these side chains and/or of Asn81 side chain might be sufficient to prevent these clashes. Furthermore, in this model, the glycan chain of both antennas can extend freely into the solvent. Overall, modeling suggests that the observed V-V homodimerization mode is also compatible with a fully glycosylated receptor.

The VC1 fragment is quite basic with a theoretical isoelectrical point of 9.8 and residues conferring this basicity are mostly located on the V domain. They form a continuous surface that covers the first β -sheet (strands A', G, F and C) as well as the B-C loop [28-29]. Interestingly, this surface is located just aside from the hydrophobic patch that forms the V-V interface in the RAGE V-V dimer model described here. Thus, RAGE self-association through its V domains would allow the two positively charged surfaces of each monomer to join into an extended basic surface as shown by analysis of the electrostatic properties of the RAGE VC1 and VC1C2 packing dimers (Fig. 3). This electropositive patch further extends onto the second β -sheet of the C1 domain with residues Lys123, Lys162, Arg216, Arg218 and Arg221, thereby creating a highly basic V-shaped platform. In the VC1C2 crystals, the C2 domain of a symmetry-related molecule interacts with the middle of this platform (Fig. 3C). Acidic residues Glu337, Glu245, Asp274 or Glu312 in the C2 domain are pointing towards the basic surface formed by the V-V packing dimer. The physiological relevance of this crystal packing interaction is questionable although it can not be excluded that it represents, e.g., an intermolecular autoinhibitory interaction of the C2 domain with the VC1 domains

from one or two other RAGE molecules, which could be an element of the inhibitory effects of sRAGE [43]. In any case, the C2 packing interaction with VC1 observed in the VC1C2 structure supports the idea that acidic ligands may be attracted by the positively charged VC1 unit. In agreement with this idea, two recently deposited structures of a complex between RAGE VC1 and DNA (RCSB entries **3S58** and **3S59**) have revealed that the DNA molecule inserts right in the center of the basic cleft in an almost perpendicular manner as compared to the RAGE dimer orientation. In addition, mutagenesis experiments performed on the mouse RAGE VC1 fragment identified key residues for heparin binding in the vicinity of the putative V-V interface and alanine mutation of hydrophobic residues located at the V-V interface showed clear binding defect for heparan sulfate, suggesting a cooperativity between RAGE V-V homodimerization and heparan binding [36]. The V domain is commonly described as the major binding site for RAGE ligands [6]. Due to its pronounced electropositive nature and because many RAGE ligands are negatively charged, it has been suggested that electrostatic interactions between such complementarily charged surfaces would govern the formation of many RAGE:ligand complexes [28]. RAGE self-association through V domain dimerization might therefore facilitate the binding of multi-modular acidic ligands to the N-terminal domains of RAGE, either within the basic cleft thereby created or on peripheral interfaces that are brought together through V-V homodimerization.

Discussion

In this paper, we report two new crystal structures for hRAGE extracellular domain: a structure of hRAGE VC1 obtained in a new crystal form and determined at 2.4 Å resolution, and the first structure of the entire hRAGE ectodomain (VC1C2) at 3.8 Å resolution. Both structures reveal that in the crystals, the hRAGE ectodomain molecules make contacts through their V domains. This property has also been observed in another crystal structure of

the apo-receptor [28], in crystal structures of the mouse RAGE VC1 fragment in complex with heparin derivatives [36], and in solution for the receptor free of ligands [32]. Whether such a V-V homodimerization can also occurs *in vivo* is still unclear. However several reports suggest that the receptor V domain plays an important role in RAGE oligomerization *in vivo* [33,36] although the precise self-association mode remains elusive. *In vitro*, stabilization of the dimeric interface has been promoted artificially either by crystal packing, cations or concentration effect [28,32]. Recently, endothelial cell surface glycans have been proposed to play a crucial role in stabilizing RAGE dimerization through V-V contacts and promoting RAGE hexamerization [36]. Since heparan sulfate is constitutively present on the cell membrane, if such a glycan-stabilized RAGE hexamer exists *in vivo*, it would most likely represents a resting form of the receptor, otherwise RAGE would be activated permanently. In that scenario, ligand binding to RAGE hexameric structures might partially or fully disrupt the RAGE:heparan interfaces - displacement of the bound glycans and/or changes in the oligomerization state of RAGE thereby inducing signal transduction. Alternatively, RAGE:heparan interfaces might be preserved while ligands bind to the hexameric structures. In that case, further oligomerization with the formation of new RAGE-RAGE interfaces might provide the mechanical force that conveys the signal through the membrane.

Thus, although V-V domain dimerization and formation of hexamers are potential modes of self-association for the RAGE ectodomain, other modes of oligomerization might be required to promote the formation of active signaling ligand:receptor complexes. RAGE self-association through C1 or C2 domains has also been reported [34-36]. Both recombinant sRAGE and the C1C2 fragment were observed to form tetramers, suggesting that V domain independent oligomerization of RAGE occurs [34]. In addition, significant C1-C1 interactions, predominantly of electrostatic nature, appear to be present in the structure of the mouse VC1 fragment [36] although they cannot be accurately analyzed due to the limited

resolution of the structure. Furthermore, the transmembrane region (residues Gly337 to Trp363) might play an important role during signal transduction. We have analyzed the sequence of RAGE TM region and found that it contains a well-conserved GxxxG motif (Fig. S6), known to promote TM helix homo-interactions [44-46]. Taken together, these results suggest that RAGE may use multiple oligomerization strategies to recognize ligands at the cell surface and transduce their signal. In particular, since it can bind to a wide variety of ligands and induce various responses, RAGE could take advantage of these different dimerization modules and use them either alternatively or simultaneously to form several oligomeric complexes differing in terms of 3D architecture and signaling capabilities. The multi-point self-association strategy we propose for RAGE is depicted schematically in Fig. 4. RAGE V-V homodimerization and further oligomerization in the presence of heparan sulfate seems to be required for the binding of various ligands including S100B, AGEs and HMGB1 [36] – ligands that all bind to the RAGE V domain. Ligand binding to other modules of the RAGE ectodomain might require the formation of different types of oligomers. RAGE self-association through C1-C1 domains [34,36], C2-C2 domains [35] and/or TM helix dimerization may be relevant in these cases and might also be used to achieve signal transduction. Whether a RAGE multimeric complex that combines all the dimerization modes discussed above at a single time exists and would signal in response to ligand binding is not known. The possibility that different dimerization schemes are used for signaling and cell adhesion must also be considered.

Apart from signaling, self-association of RAGE molecules on the cell surface would create a patch of oligomers that may be particularly relevant for the recruitment of leukocytes via RAGE interaction with the I-domain of integrin $\alpha_M\beta_2$ [15,47]. A common feature of many adhesion molecules is their tendency to assemble into dense clusters at the junctions to facilitate cell-cell contacts. Interestingly, such an oligomerization model has been proposed

for the interaction between ICAM-1 and the related integrin $\alpha_L\beta_2$, during synapse formation [48]. Thus, an evident parallel can be drawn between RAGE and ICAM-1, and formation of a long, uni- or bidirectional RAGE cluster according to the model proposed in Fig. 4 could be crucial to engage with the $\alpha_M\beta_2$ integrin molecules presented on leukocytes. In any case, further structural studies will be necessary to fully comprehend the dynamic mechanisms that govern RAGE-mediated ligand recognition, cell signaling and adhesion processes.

Experimental Procedures

Expression and purification of hRAGE domains

The DNA sequences coding for hRAGE VC1 (residues Ala23 to Glu231) and hRAGE VC1C2 (residues Ala23 to Pro323) were amplified from human cDNA (generous gift from Dr. Klaus Hvid Nielsen) and cloned in between the *NcoI* and *XhoI* restriction sites of vector pETM11. The resulting pETM11-VC1 and pETM11-VC1C2 constructs were transformed in Shuffle T7 Express *E. coli* cells (New England Biolabs, Ipswich, MA, USA) to enhance disulfide bond formation. Cells were grown at 37°C in 2YT medium supplemented with kanamycin (50 µg/ml) and expression of hRAGE domains was induced for 16h at 18°C by the addition of 1 mM IPTG. Cells were harvested by centrifugation (20 min. at 6000 rpm and 4°C). Each 2L culture pellet was resuspended in 50 mL of Buffer A (50 mM HEPES pH 7.5, 300 mM NaCl, 30 mM imidazole, 1 mM PMSF) and the cells were opened by sonication. After clarification by centrifugation (20 min. at 16000 rpm and 4°C), the supernatant was applied onto a 5 mL Ni-column (HisTrap FF column from GE Healthcare Life Sciences, Uppsala, Sweden). After high salt wash (50 mM HEPES pH 7.5, 1 M NaCl, 30 mM imidazole, 1 mM PMSF) to remove unspecifically bound proteins, the His₆-tagged proteins were eluted with Buffer B (50 mM HEPES pH 7.5, 300 mM NaCl, 500 mM imidazole, 1 mM

PMSF). The His₆-tag was then removed by overnight incubation at 4°C of the protein with a 1:30 (rTEV:protein) mass ratio of house-made recombinant TEV (His₆-rTEV) in buffer D (50 mM HEPES pH 7.5, 300 mM NaCl, 0.5 mM EDTA). The cleaved proteins were further purified by reloading them onto the HisTrap column. Due to some non-specific binding of the hRAGE domains to the Ni-column, the cleaved products eluted both in the flow-through and in the high salt wash fractions, while contaminants with strong Ni-affinity, uncleaved protein, and His₆-rTEV remained bound to the column. The protein samples were then concentrated and rediluted with 50 mM HEPES pH 7.5 to reach a final salt concentration of 180 mM NaCl for VC1C2 and 250 mM NaCl for VC1. The samples were then applied onto a 9 mL Source 15S cation exchange column (GE Healthcare Life Sciences). Elution was performed with a 100 mL linear gradient from 180 mM to 600 mM NaCl for VC1C2 and from 250 mM to 650 mM NaCl for VC1. The hRAGE VC1C2 domain eluted as a single peak around 380 mM NaCl (Fig. S3) while hRAGE VC1 domain eluted as a single peak around 440 mM NaCl. Fractions containing the target protein were analyzed by SDS-PAGE, pooled, flash-frozen in liquid nitrogen and stored at -80°C until use. Homogeneity of the samples was checked by running a small aliquot of the hRAGE domains preparation onto a 24 mL Superdex 75 column (GE Healthcare Life Sciences) equilibrated in 25 mM HEPES pH 7.5, 250 mM NaCl. Both hRAGE VC1C2 and hRAGE VC1 eluted as a sharp, single peak consistent with a monomeric protein of respectively 33 and 24 kDa (Fig. S3).

Crystallization of hRAGE VC1 and VC1C2 domains

Prior to crystallization experiments, the buffer composition of both hRAGE VC1C2 and VC1 samples was adjusted to 25 mM HEPES pH 7.5, 250 mM NaCl. The proteins were subsequently concentrated to 8 mg/ml. Initial crystallization experiments were carried out in 96-wells sitting drop plates using a MOSQUITO robot (TTP LabTech, Melbourne,

Hertfordshire, UK) and commercial screens from Hampton Research (Aliso Viejo, CA, USA) and Molecular Dimensions Ltd (Newmarket, Suffolk, UK). For hRAGE VC1, crystals appeared overnight at 4°C over a reservoir containing 2.5 M Na acetate pH 7.5. For data collection, the crystals were cryoprotected by soaking into the reservoir solution supplemented with 25% w/v sucrose followed by flash cooling in liquid nitrogen. For hRAGE VC1C2, spherulites appeared after a few days at 4°C using 0.1 M Tris pH 8.5, 20% ethanol as the precipitant solution. Better crystals were obtained using a reservoir solution containing 0.1 M Tris pH 8.0, 10% ethanol plus 150 mM NaCl added after mixing with the protein. Prior to data collection, these crystals were cryoprotected by soaking into the reservoir solution supplemented with 38-42% ethylene glycol followed by flash cooling in liquid nitrogen.

Data collection and structure determination

Datasets for hRAGE VC1 were collected at 100K on the 911-2 beamline at Max-Lab (Lund, Sweden) and processed with XDS [49] to 2.4 Å resolution (Table I). hRAGE VC1 crystals displayed an apparent P₆₂22 symmetry with one molecule in the asymmetric unit. The structure was solved by MR in PHASER [50], using the VC1 moiety of the MBP-VC1 structure (RCSB entry **3O3U**) [29]. After the first rounds of refinement using PHENIX.REFINE [51], the R-factors values were however still high despite the fact that the model was almost complete. We hypothesized that a non-perfect symmetry of the two molecules forming the VC1 dimer impaired full refinement in space group P₆₂22. The VC1 dataset was therefore reprocessed in the lower symmetry space group P₆₂ with two molecules per a.u. and refinement in P₆₂ proceeded smoothly (Table I). Refinement of the model was carried out by alternating between cycles of manual rebuilding in COOT [52] and cycles of energy minimization with PHENIX.REFINE [51] using individual ADP refinement as well as TLS refinement. Datasets for hRAGE VC1C2 were collected at 100K on the X06SA

beamline at SLS (PSI, Villingen, Switzerland) and processed with XDS [49]. hRAGE VC1C2 crystals belonged to space group $P6_5$ with 2 molecules per asymmetric unit and diffracted to a maximal resolution of 3.8 Å. MR was performed in PHASER [50] using a search model encompassing the entire VC1 dimer described above. This gave a clear molecular replacement solution (Z -score>20) and initial density maps revealed an additional density for one of the two missing C2 domains (Fig. S1). Refinement of the model was carried out by alternating cycles of manual rebuilding in COOT [52] and cycles of energy minimization with PHENIX.REFINE [51] using group ADP refinement as well as TLS refinement and imposing tight restraints on the geometry based on our model for the VC1 moiety obtained at higher resolution. The second C2 domain could not be seen in the electron density and was therefore not modeled (Fig. S1). The quality of both structures was assessed with *MOLPROBITY* [53]. All figures were made with the Pymol Molecular Graphics System, version 0.99rc6, DeLano Scientific LLC.

Accession codes

The coordinates and structure factors for the hRAGE VC1 and hRAGE VC1C2 structures have been deposited in the RCSB with the accession codes 4LP4 (hRAGE VC1) and 4LP5 (hRAGE VC1C2).

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Supporting information

Supplementary information is available online and contains the following figures:

Fig. S1: Initial electron density maps showing the density for one of the C2 domains in the hRAGE VC1C2 crystals and comparison of the crystal packing around the positions of the two C2 domains.

Fig. S2: Structural alignment of all available structures for the hRAGE VC1 module and overall topology of the two Ig domains.

Fig. S3: Chromatograms and SDS-PAGE analysis of the hRAGE VC1C2 sample.

Fig. S4: Comparison of the previously reported hRAGE VC1 structure and mRAGE VC1 structure with our VC1 and VC1C2 dimer models.

Fig. S5: Model for RAGE glycosylation on residue Asn81 and compatibility with a V-V homodimer.

Fig. S6: Putative RAGE homodimerization through its transmembrane region.

Figure Legends

Fig. 1: The crystal structure of the hRAGE ectodomain. **(A)** Overview of the hRAGE VC1C2 structure determined at 3.8 Å resolution. **(B)** Surface representation of the hRAGE VC1C2 monomer colored according to its overall electrostatic potential calculated with APBS [54]. Surface areas colored blue are positively charged and areas in red are negatively charged. **(C)** Topology diagram for the C2 domain. **(D)** General overview of hRAGE C2 domain from the VC1C2 structure with the β -strands labeled according to the canonical Ig fold nomenclature [4]. The two cysteines forming the intramolecular disulfide bridge are shown as green sticks.

Fig. 2: The structurally conserved V-V domain crystal packing dimer. **(A)** Final electron density map ($2mF_o - DF_c$ map contoured at 1σ , blue mesh) and final model for the interface

region of the hRAGE VC1 crystal packing dimer. **(B)** Close-up view on the residues at the V-V domain interface from the hRAGE VC1 structure. Residues stabilizing the interface are shown as sticks, and bridging water molecules as red spheres. **(C)** Superimposition of the hRAGE VC1 (monomer A in light blue, monomer B in cyan) and VC1C2 (monomer A in magenta, monomer B in dark violet) packing dimers. The superimposition was made by aligning the VC1 moiety of monomer A from each structure. The flexibility of the V-V packing dimer interface is relayed to the rest of the molecule as reflected by the respective positions of the C1 C-terminal residue Glu231 in each structure. The eye and arrow indicate the view in panel **(D)** and the dotted line represents the plan of the view in panel **(D)**, perpendicular to the view in panel **(C)**. **(D)** Close-up view on the V-V packing dimers from each structure. The flexibility of the interface arises from a swing movement in between the two monomers indicated by red arrows.

Fig. 3: Electrostatics properties of the hRAGE VC1 dimer formed through crystal contacts. **(A)** Surface representation of the hRAGE VC1 packing dimer colored according to its overall electrostatic potential calculated with APBS [54]. Surface areas colored blue are positively charged and areas in red are negatively charged. **(B)** Same as (A) but viewed from the top. Basic residues within the V-shaped platform are indicated for one of the two monomers. **(C)** Surface representation of the hRAGE VC1C2 packing dimer colored according to the electrostatic potential. The sole C2 domain that could be traced in the VC1C2 structure packs into the V-V dimer formed between two symmetry-related molecules (shown in green) along its electronegative surface. Another C2 domain (light green) from a symmetry-related molecule packs into the V-V basic domain groove.

Fig. 4: RAGE multimodal oligomerization strategy. Schematic model representing all the possible RAGE oligomerization modes discussed here. Dotted arrows marked with numbers indicate the regions where flexibility can arise due to the presence of flexible linkers or through the formation/dissociation of the various dimerization interfaces. 1) Formation/disruption of the V-V dimer interface. This interaction appears to be further stabilized by ligand binding (heparin [36], DNA). 2) Flexibility due to the C1-C2 linker. 3) C1-C1 homodimerization by β -strand swapping or non-covalent electrostatic interactions as observed for the mouse VC1 fragment in the presence of heparin [36]. 4) Flexibility due to the long C2-TM linker (17 residues from Ile321 to Gly337). 5) Homodimerization through the TM segments. 6) Out of plane oligomerization enhanced by heparan sulfate binding (hexamerization) [36] and possibly other ligands.

Table I: data collection and refinement statistics.

A **B** **C** **D**

A **B** **C** **D**

A **B** **C**

ADHESION

LIGANDS

ADHESION

Supporting Information

Structural insights into the oligomerization mode of the human Receptor for Advanced Glycation End-products (RAGE)

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CONTENT

- Fig. S1** Initial electron density maps showing the density for one of the C2 domains in the hRAGE VC1C2 crystals and comparison of the crystal packing around the positions of the two C2 domains.
- Fig. S2** Structural alignment of all available structures for the hRAGE VC1 module and overall topology of the two Ig domains.
- Fig. S3** Chromatograms and SDS-PAGE analysis of the hRAGE VC1C2 sample.
- Fig. S4** Comparison of the previously reported hRAGE VC1 structure [1] and mRAGE VC1 structure [4] with our VC1 and VC1C2 dimer models.
- Fig. S5** Model for RAGE glycosylation on residue Asn81 and compatibility with a V-V homodimer.
- Fig. S6** Sequence alignment for RAGE transmembrane/juxtamembrane regions and model for TM-helix homodimerization.

Fig. S1: Crystal packing around the C2 domains in the hRAGE VC1C2 crystals. **(A)** Initial electron density maps obtained after MR with our hRAGE VC1 dimer as a search model showing an additional density corresponding to one of the two C2 domains. The 2mF_o-DF_c map (blue mesh) and the mF_o-DF_c map (green mesh) are respectively contoured at 1 and 3σ. **(B)** Crystal packing around the first C2 domain in the final model. Tight packing against symmetry-related molecules prevents free movement of the domain. **(C)** and **(D)** Final electron density maps and final model centered on the solvent pocket where the second C2 domain is likely to be located. The 2mF_o-DF_c map (blue mesh) and the mF_o-DF_c map (green and red mesh) are respectively contoured at 1 and 3σ. Due to the high degree of mobility

between the VC1 and C2 domains that is conferred by the presence of an unstructured C1-C2 linker, the second C2 domain can move freely in the large solvent void indicated by the oval shape in panel D and therefore does not adopt a fixed position.

Fig. S2: Comparison of all available structures for the hRAGE VC1 moiety and general topology of the two Ig domains. **(A)** Structural alignment of the hRAGE VC1 part of our VC1 dimer structure (red), of our VC1C2 dimer structure (magenta), of the apo-VC1 structure (green) [1] and of the MBP-VC1 structure (blue) [2]. The regions of divergence between these four structures are indicated by blue arcs and the width of the arc reflects the degree of divergence. **(B)** General topology of the V and C1 domains according to the canonical nomenclature of Ig domains [3].

Fig. S3: Elution profiles and SDS-PAGE analysis of the purity and homogeneity of the hRAGE VC1C2 sample. **(A)** Elution profile of hRAGE VC1C2 on the Source 15S column. The protein elutes as a single peak around 380 mM NaCl. **(B)** SDS-PAGE analysis of the peak fractions from the Source 15S run displayed in (A). hRAGE VC1C2 migrates as two bands (40 and 33 kDa, a and b) in reducing conditions, due to a non-complete reduction of the 6 cysteines forming disulfide bridges. **(C)** Elution profile of hRAGE VC1C2 on the Superdex 75 column. The protein elutes as a single, sharp peak consistent with a monomeric protein.

Fig. S4: A structurally conserved V-V domain dimer derived from crystal packing. **(A)** Crystal packing in the hRAGE VC1 structure published by Koch *et al.* [1] revealing two possible modes of homodimerization. In the ‘Dimer 1’ model proposed as physiologically relevant by Koch *et al.* [1], the two RAGE molecules pack against each other along the C1 domain, in a parallel manner, and the C1-C1 interface is stabilized by Zn^{2+} ions. **(B)** Second possible mode of RAGE homodimerization extracted from the hRAGE VC1 structure by Koch *et al.* [1], which is similar to the V-V domain dimerization we observe in our two structures. **(C)** VC1 dimer model for mouse RAGE extracted from the mouse-VC1:heparan sulfate complex structure by Xu *et al.* [4]. **(D)** Comparison of the VC1 dimers observed in all these structures.

Fig. S5: Model for RAGE glycosylation on residue Asn81 and compatibility with a V-V homodimer. **(A)** Glycosylated model of a RAGE V-V homodimer. The RAGE VC1 monomer was glycosylated *in silico* (Glyprot program [5]) on Asn81 with a branched glycan encompassing 7 carbohydrate moieties according to the glycosylation pattern determined by Hanford *et al.* [6] for murine RAGE. The glycosylated version of RAGE VC1 was then superimposed onto both monomers of the VC1 packing dimer to reconstruct a glycosylated homodimer. **(B)** Zoomed view on the glycan chain of Asn81. The residues from the other VC1 monomer that clash with the carbohydrates are indicated as sticks and are labeled. The directions through which the glycan chain might extend are indicated with arrows.

A

B

Fig. S6: Putative RAGE homodimerization through its transmembrane region. (A) Sequence alignment for the RAGE transmembrane domain and the short region following the TM helix (juxtamembrane region). The alignment was performed with MULTIALIN [7] and the sequence conservation analysis was done in ALINE [8]. The transmembrane region (residues 337 to 363 for human RAGE) is indicated by a helix above the sequences. The consensus

sequence prone to promote helix-helix dimerization within the membrane is shown at the bottom of the figure with the two glycines supposedly forming the GxxxG motif shown in red. **(B)** Model for hRAGE TM helix homodimerization based on the NMR structure of the TM helix dimer of GlpA [9] and on the NMR structure of hRAGE C-terminal region encompassing residues 363 to 376 [10]. Residues were attributed by aligning the ³⁴²ALxxGIxxGLxxA³⁵⁴ sequence of hRAGE to the ⁷⁵LIxxGVxxGVxxT⁸⁷ sequence of GlpA. This allowed the construction of a helix model covering residues Gly337-Arg365 after which the NMR structure of hRAGE C-terminal tail (Trp363 to Pro376) was superimposed to give the final model.

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