UNIVERSITY OF CALIFORNIA, SAN DIEGO

Structural Characterization of RAGE-Heparan Sulfate Interactions and RAGE Oligomerization

A thesis submitted in partial satisfaction of the requirements for the degree

Master of Science

in

Biology

by

Jeffrey Hao-Wei Young

Committee in charge:

Professor Jeffrey D. Esko, Chair
Professor Randolph Hampton, Co-Chair
Professor Russell Doolittle
Professor Partho Ghosh

2013

The Thesis of Jeffrey H Young is approved and it is accepted in quality and form for publication on microfilm and electronically:

_____

_____

_____

Co-Chair

_____

Chair

University of California, San Diego

2013

DEDICATION

To:

Ruling Liu, my loving mother

Chih Hong Young, my supportive father

and

Liu Chien Li Su, in memory of my maternal grandmother

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF SUPPLEMENTARY FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to foremost thank Dr. Jeffrey D. Esko for his support as my advisor and as the committee chair. The opportunity to work in his lab was decisively one of the most pivotal decisions in my science academia. As always, Dr. Esko has shown me the remarkable attributes of understanding science by challenging me to shift from a linear approach to a complex perspective when analyzing experimental data. I am grateful for his simply gestures of "hello", thoughtful conversations, humorous stories, and appreciation for appropriately advising me how to balance life. His intellectual understanding of glycans is truly remarkable. Dr. Esko's support, advice, and teaching have further reinforced my passion in science.

I would also like to sincerely thank Dr. Ding Xu for serving as my mentor and as a respectable researcher. Since my first day in the Esko lab, his persistence, patience, and guidance have trained me to become more meticulous in scientific experiments, enhance my critical perception of mistakes, and develop my appreciation for protein structural analysis. By constantly nurturing my interest in science, Dr. Xu has enriched me with a profound understanding of biochemical techniques. Ding's profound understanding of biochemistry and his knowledgeable insight in experiments is truly at the highest caliber. The field of scientific research could have never offered me a finer mentor. Thank you.

I would like to thank Dr. Partho Ghosh, Dr. Randy Hampton, and Dr. Russell Doolittle for serving as members of the committee. I am grateful for Dr. Ana M. Pajor by providing me the opportunity to begin research through her laboratory. Her disciplined methods and advice has appropriately guided me to conduct proper experiments, develop organization skills, and record appropriate data. Thank you.

Last but not least, I'd like to thank all the wonderful members of the Esko lab for their constant help, the non-stop amusing jokes, and the educational environment.

Work presented in this thesis will be submitted for publication with authors Ding Xu and Jeffrey D. Esko. Work was conducted within the department of Cellular and Molecular Medicine, and the Glycobiology Research and Training Center, University of California, San Diego, La Jolla, California.

ABSTRACT OF THE THESIS

Structural Characterization of RAGE-Heparan Sulfate Interactions and RAGE Oligomerization

by

Jeffrey Hao-Wei Young

Master of Science in Biology

University of California, San Diego, 2013

Professor Jeffrey D. Esko, Chair

Professor Randolph Hampton, Co-Chair

The receptor for advanced glycation end products, RAGE, is a protein receptor localized on the cellular plasma membrane which binds to various ligands such as AGEs or HMGB1. RAGE activates downstream inflammatory signaling associated with diabetes and atherosclerosis. Our recent studies suggest heparan sulfate, a negatively charged linear polysaccharide, directly interacts with RAGE to promote receptor signaling and oligomerization.

The objective of this study is to characterize the structural details of RAGE-heparan sulfate interactions and RAGE oligomerization. Site directed mutagenesis, heparin sepharose chromatography, and

filter binding assays shows that basic residues on the V domain (K39, K43, K44, R104, and K107) and C1 domain (R216 and R218) of RAGE are essential for binding to heparin and heparan sulfate.

Gel filtration chromatography demonstrated heparin-derived oligosaccharides with a minimal length of a dodecasaccharide (12-mer) are capable of inducing a stable tetramer of RAGE. Further testing suggests the tetramer complex consists of four RAGE monomers with two dodecasaccharides.

A second site directed mutagenesis study identifies a patch of hydrophobic residues on the V domain (V35, V78-L79 and F85-V86) affecting RAGE oligomerization. Our findings show critical residues important for heparan sulfate binding and a subset of hydrophobic binding residues necessary for RAGE oligomerization. The construction of a preliminary crystal structure of RAGE with heparin-derived oligosaccharide is in progress.

Lastly, the heparan sulfate and hydrophobic binding surface of RAGE appears to be separate from its ligand binding surface based on a ligand binding assay. Thus, understanding these RAGE-heparan sulfate interactions could lead to the development of pharmacological agents reducing the progression of certain pathological conditions in cardiovascular diseases and diabetes.

INTRODUCTION

RAGE

The receptor for advanced glycation end products (RAGE) is a multi-ligand receptor expressed in lung alveolar cells, neurons, epithelial cells, leukocytes, and endothelial cells [1]. RAGE has been shown to bind to various ligands such as advanced glycation end products (AGEs), S100 proteins, amyloid B, and high mobility group protein box 1 (HMGB1) which have been linked to diabetic complications, inflammatory related diseases, Alzheimer's disease, neurodegenerative disorders, and cancer [2]. This protein receptor activates a downstream pro-inflammatory signaling cascade, although the mechanism behind the coupling of receptor-ligand interactions and signaling is still unknown [3].

Structural insights of RAGE

As the name suggests, RAGE was originally identified as a receptor for AGEs. RAGE is a type I transmembrane protein receptor consisting of 404 amino acids and consists of three extracellular domains, V, C1, and C2, a transmembrane helix, and a short intracellular negatively charged C-terminal tail [4]. Studies have shown V and C1 domains are primarily responsible for ligand binding, while the short cytoplasmic tail is essential for signal transduction by recruiting adaptor proteins such as Dia1 or the kinase, Erk1 [5]. At a size of approximately 35 kDa, RAGE possesses several isoforms including those lacking the transmembrane and signaling domain referred to as soluble RAGE (sRAGE) [6]. Previous studies have shown that sRAGE is capable of antagonizing RAGE signaling both in vitro and in vivo which could be used as a potential therapeutic agent for cardiovascular diseases [7]. RAGE signaling initiates the activation of NF-kB and MAPK pathways leading to the expression of pro-inflammatory cytokines responsible for many pathological diseases [5]. We have recently demonstrated RAGE signaling is dependent upon heparan sulfate [8].

Heparan sulfate

Heparan sulfate, a type of glycosaminoglycan, is a linear sulfated polysaccharide found on the cell surface and the extracellular matrix in all animal tissues [9]. Playing multiple roles in vascular biology and inflammation, heparan sulfate often serves as a co-receptor for signaling by facilitating the formation of a

receptor-ligand complex [10]. Heparan sulfate serves as the co-receptor for fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), simultaneously binding both ligands and receptors and improving the duration and amplitude of signaling [11 12].

Recently, we have shown that signaling of RAGE ligands such as AGE and S100 proteins depend upon heparan sulfate [8]. An immunoblot analysis of ERK phosphorylation in human microvascular endothelial cells demonstrated stimulation by various RAGE ligands. Results showed pre-treating these endothelial cells with heparin lyase induced loss of RAGE signaling. Our study aims to identify the specific residues on RAGE critical for heparan sulfate binding. Thus, reducing the signaling of many RAGE ligands by targeting heparan sulfate-RAGE interactions with potential antagonists could alter the progression of diseases.

RAGE oligomerization

Heparan sulfate also plays a key role in RAGE oligomerization by inducing the formation of RAGE oligomers. Past studies have shown that RAGE functions as homodimers on the plasma membrane, a phenomenon that may contribute to ligand recognition and signal transduction [13]. Dimerization of RAGE is an important step in receptor signaling following ligand binding, as shown by MAPK phosphorylation and downstream transcriptional activation [12]. Our recent studies indicated RAGE oligomerization and signaling depends on its interaction with heparan sulfate. RAGE oligomerization is a prerequisite for signal transduction, but the mechanism of RAGE oligomerization and the organization of the oligomers bound to heparan sulfate remains unknown. We aim to identify certain RAGE residues important in RAGE oligomerization.

Signaling of RAGE ligands depend on heparan sulfate

HMGB1 binds to heparin and heparan sulfate; however, mutagenesis studies have shown that signaling by HMGB1 can be uncoupled from its capacity to bind heparan sulfate [8]. Nevertheless, cell surface heparan sulfate remains a requirement for HMGB1 signaling because of the direct interaction between heparan sulfate and RAGE, which suggests heparan sulfate is a prerequisite for RAGE mediated

signaling. Based on this finding, we predicted that the signaling of other RAGE ligands such as AGEs and S100 proteins might also depend on heparan sulfate, even though many of them are not heparin binding proteins. Some of our recent experiments have demonstrated RAGE ligands such as AGE and S100A also depend on heparan sulfate for signaling, as pretreatment of endothelial cells with heparan lyases rendered them unresponsive to all ligands tested. This important finding provides a basis for targeting heparan sulfate/RAGE interactions as a way to potentially halt the progression of inflammatory related conditions, because the development of specific antagonists would affect the signaling of RAGE ligands produced in a disease state.

MATERIALS AND METHODS

Mutagenesis of RAGE

Mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Agilent). The RAGE mutants were prepared using sRAGE-pET45b as the template and a method modified from Agilent Quick-change site-directed mutagenesis kit. Mutants were confirmed by sequencing and expressed in Origami-B cells. Mutants were purified first by Ni-Sepharose 6 fast flow column, then subjected to heparin sepharose chromatography using a HiTrap heparin high performance column (1ml, GE Healthcare).

***E.coli* expression of sRAGE**

In experiments employing mutants of RAGE, recombinant protein was generated in *Escherichia coli*. The extracellular domain (Ala-23–Glu-326) of RAGE (Open Biosystems) was cloned into pET45b (Novagen) using the *Psh*A I site. Expression was carried out in Origami-B cells (Novagen) carrying the pGro7 (Takara) plasmid expressing chaperonin proteins GroEL and GroES of *E. coli*. Transformed cells were grown in LB medium supplemented with 12.5 mg/ml tetracycline, 15 mg/ml kanamycin, 35 mg/ml chloramphenicol, and 50 mg/ml carbenicillin at 37°C. When the absorbance at 600 nm reached 0.4–0.7, isopropyl-D-thiogalactopyranoside (0.15 mM) and L-arabinose (1 mg/ml) were added to induce the expression of RAGE and chaperonin proteins, respectively. The cells were allowed to shake overnight at 22 °C. Purification was performed by using a Ni-Sepharose 6 fast flow column followed by gel filtration chromatography with Superdex 200 10/300.

Nitrocellulose filter binding assay

Recombinant wild type sRAGE (1 ug) and mutants (1 ug) were incubated with 10,000 counts of [^{35}S] labeled microvascular endothelial lung heparan sulfate in 100 µl PBS for one hour at 22°C. 1 µg of BSA (<0.5% of retention) and FGF2 (~50% retention) were used as the negative and positive control, respectively. Binding reaction was applied onto a Bio-Rad 96 well blot apparatus with a pre-washed nitrocellulose membrane and washed with 100 µl PBS twice. With the vacuum on, the whole binding reaction was applied into individual wells and was absorbed onto the membrane. [^{35}S] heparan sulfate was

passed through the membrane and samples bound to RAGE protein were essentially retained. After the complexes were absorbed, the membrane was washed, individually cut into small squares, and eluted with 500 µl of HEPES buffer containing 1M NaCl for 10 min at 22°C. Scintillation fluid was then added and counts were measured by scintillation counting. The counts of [^{35}S] heparan sulfate in the eluent were measured by scintillation counting. The extent of binding was then quantified by dividing the membrane retained radioactivity by the total input radioactivity.

RAGE tetramer gel filtration chromatography.

Recombinant purified wild type sRAGE (200 ug) and mutants (200 ug) were incubated overnight with a 16-mer, heparin-derived oligosaccharide (Iduron). A Superdex 200 (10/300 mm) gel filtration column was calibrated with molecular protein weight standards for reference. Profile runs of each RAGE mutant was measured and traced.

RAGE ligand binding assay

Recombinant wild type sRAGE (200 ng) and mutants (200 ng) were coated onto a 96 well plate in 100 ul PBS overnight at 4 C. After multiple washes with PBS and loading of blocking buffer, biotinylated HMGB1 (0, 50, 100, 250, 500, and 1500 ng) was applied to individual wells and incubated for one hour. After incubation, 100 ul of Streptavidin-HRP was applied for 15 minutes. The wells were washed 7 times with 100 ul PBS. Turbo TMB-ELISA substrate (50 ul, Thermo Scientific) was then loaded for 10 minutes and reaction was stopped with 50 ul sulfuric acid. Results were then measured by absorbance and data was analyzed by using Prism software. The mean equilibrium dissociation constant (K_D) was measured using non-linear regression analysis.

RESULTS

Identification of RAGE residues critical for heparin binding

Based on the interaction of heparin with other proteins, we surmised that binding of heparan sulfate to RAGE would involve electrostatic interactions of lysine and arginine residues of RAGE with sulfate and carboxyl groups in the heparan sulfate chains. A distinct feature of RAGE is that it contains a large number of surface exposed basic residues. Interestingly, almost all the basic residues are localized to V and C1 domains, whereas the C2 domain contains only one lysine and one arginine residue. The thirteen lysine and sixteen arginine residues in the V and C1 domains are segregated into two groups that run parallel to the long axis of the protein (Figure 1). Theoretically, both surfaces of RAGE have the potential to form heparan sulfate binding sites. Several basic residues located at surface II in the V domain have been shown to be involved in binding to AGE-type ligands and mediate S100B binding [14].

To determine the heparan sulfate binding sites of RAGE, a site directed mutagenesis study was performed to map out these important residues. Evolutionary conserved lysine (K) and arginine (R) residues in the V and C1 domain of RAGE were screened and selected for as the most appropriate residues to mutate. A sequence alignment of RAGE across ten mammalian species (RAGE is only present in mammals) suggested twenty two basic residues (Figure 2). Initially, each residue was mutated to alanine (A) and further pairs or sets of mutants were combined. Heparin Sepharose chromatography showed seven RAGE mutants displaying a 60 to 120 mM reduction in salt concentration when compared to wild type sRAGE (Table 1). Out of these seven RAGE residue mutations, five were found in the V domain (K39, K43, K44, R104, and K107) and two were located in the C1 domain (R216 and R218) of RAGE (Figure 3). The remaining fifteen lysine and arginine RAGE mutations did not affect heparin binding. Thus, seven RAGE basic residues (K39, K43, K44, R104, K107, R216, and R218) seem to be critically important for heparan sulfate interaction.

RAGE mutants affect binding to heparan sulfate

To determine whether the identified heparin binding mutations also affected RAGE binding to heparan sulfate, we performed a filter-binding assay on the RAGE mutants: K39A, K43A-K44A, R104A,

K107A, and R216A-R218A. Recombinant produced wild type sRAGE and mutants were combined with [^{35}S] heparan sulfate from endothelial cells. A filter binding assay measured the level of binding retained between [^{35}S] heparan sulfate and RAGE. Results indicated the seven RAGE residue mutations with reduced binding to heparin Sepharose also showed reduced binding to heparan sulfate (Figure 4). Similar to heparin Sepharose chromatography, RAGE with paired double mutations demonstrated the greatest reduction in heparan sulfate binding. Thus, K39, K43-K44, R104, K107, and R216-R218 are the basic residues of RAGE critical for both heparin and heparan sulfate binding.

Heparin induces tetramerization of sRAGE

One mechanism that heparan sulfate might contribute to RAGE signaling is by inducing RAGE oligomerization. Receptor oligomerization is a common phenomenon in signal reception, and heparan sulfate can induce oligomerization of other types of receptors such as FGF and VEGF [12]. To test whether heparan sulfate played a similar role in RAGE reception, we mixed recombinant sRAGE with heparin-derived oligosaccharides of various sizes. Recombinant sRAGE is monomeric in solution at a concentration as high as 0.6 mM. Interestingly, all monomer was converted to tetrameric form when mixed with oligosaccharides longer than dodecamer as demonstrated by gel filtration chromatography (Figure 5A). The induction of RAGE oligomerization by oligosaccharide can be achieved at a protein concentration as low as 10 μM (lowest tested). In addition, the purified tetrameric complex of RAGE with the oligosaccharide is very stable as seen by a repeated chromatography of the complex a week later.

Based on the elution position and the amount of bound oligosaccharide in the heparan sulfate-RAGE complex, we estimate the stoichiometry of the complex to be 4 RAGE monomers with 2 heparin oligosaccharides (Figure 5B). Combining a 4:1 molar concentration of sRAGE to 16-mer demonstrates two roughly equal peaks at the predicted tetramer and monomer position of a gel filtration chromatography. This suggests half of the sRAGE was capable of being converted to tetramers when mixed with the heparin-derived oligosaccharide while the other half remained as a RAGE monomer. By increasing the amount of oligosaccharide to a 2:1 molar ratio of sRAGE to 16-mer, sRAGE completely converted to tetramer.

Heparan sulfate binding RAGE mutations affect oligomerization

How RAGE oligomerization is regulated remains unknown. We decided to test whether the RAGE mutations critical for heparan sulfate binding was also important in RAGE oligomerization. When mixed with the heparin derived oligosaccharide, the seven RAGE mutants affecting heparan sulfate binding displayed a substantial reduction (70% to 100% when compared to wild- type RAGE) in tetramer formation suggesting that same amino acids critical for heparin binding were also significant for RAGE tetramerization (Figure 6). Thus, the same seven mutant RAGE basic residues which affected heparan sulfate binding (K39, K43-K44, R104, K107, and R216-R218) also disrupted RAGE oligomerization indicated by gel filtration chromatography. In contrast, the remaining fifteen RAGE residue mutants that did not affect heparin binding were able to still form a tetramer complex.

A RAGE hydrophobic patch region mediates RAGE oligomerization

RAGE oligomerization has been shown to demonstrate an energetic advantage for the binding of ligands to the V domain of RAGE [15]. The receptor is also capable of binding to another RAGE molecule, sRAGE, or the V domain peptide, which harbors the key region mediating RAGE homo-interaction. A previous study demonstrated hydrophobic interactions are a major driving force for RAGE oligomerization [13]. Twelve selected hydrophobic residues (V35, W61, V63-L64, W72, V78-L79, F85-L86, L159, and L164-V165) from a patch on the V domain of RAGE were assessed by site directed mutagenesis (Table 2). Each residue or pair of residues was mutated to alanine. RAGE mutants were then incubated with heparin derived oligosaccharides (16-mer) and resolved by gel filtration chromatography. Results showed V78A-L79A and F85A-V86A, when combined with 16-mer, disrupted RAGE oligomerization by preventing complete formation of the tetramer (Figure 7A). In contrast, the remaining eight RAGE hydrophobic patch mutants did not affect oligomerization sharing similar profiles with oligosaccharide mixed wild type RAGE. Thus, V78A-L79A and F85A-V86A affect receptor oligomerization with heparin-derived oligosaccharides suggesting these hydrophobic residues are located on the oligomeric interface of RAGE (Figure 7B).

Further experiments showed that none of the three identified RAGE hydrophobic residue mutants affecting oligomerization had any effect on binding with heparin (Figure 8). Based on heparin Sepharose chromatography, V78A-L79A and F85A-L86A shared similar salt concentration elution gradients with wild type RAGE. In contrast, R216A-R218A was previously shown to have a reduced salt concentration elution gradient when compared to wild type RAGE. Therefore, the hydrophobic patch of RAGE does not interfere with the binding sites of heparin and presumably heparan sulfate.

We have been attempting to solve the co-crystal structure of RAGE VC1 domain tetramer in complex with dodecasaccharide in collaboration with Dr. Lars Pedersen (National Institute of Environment Health Sciences). We were able to obtain a crystal from the purified RAGE VC1 oligosaccharide complex that diffracted at 3.4 Å; however, the oligosaccharide was missing from the crystal possibly due to high salt concentration (300 mM) needed for crystallization. Nevertheless, we were able to identify RAGE dimers in the crystal lattice (Figure 9). This dimer interface includes all the hydrophobic residues identified by mutagenesis critical for RAGE oligomerization.

RAGE ligand, HMGB1, binding unaffected by heparan sulfate and hydrophobic binding sites of RAGE

Having demonstrated that the binding sites for heparan sulfate on RAGE are essential for oligomerization, but the hydrophobic residues critical for oligomerization did not affect heparan sulfate binding; we concluded that heparan sulfate binding and the oligomeric interface appear to be on different surfaces. We next wanted to test if either these basic residues or hydrophobic patch mutants of RAGE had any effect on ligand binding. Based on ligand binding tests, the heparan sulfate and hydrophobic residues did not have an impact on binding with HMGB1, a RAGE ligand (Figure 10). Further ligand tests with S100b protein, another RAGE ligand, showed similar results. Therefore, these findings indicate heparan sulfate and hydrophobic binding residues do not interfere with RAGE ligand suggesting a different surface on RAGE for ligand binding.

Work presented in this thesis will be submitted for publication with authors Ding Xu and Jeffrey D. Esko. Work was conducted within the department of Cellular and Molecular Medicine, and the Glycobiology Research and Training Center, University of California, San Diego, La Jolla, California.

DISCUSSION

RAGE is a multi-ligand receptor on vascular cells and has been shown to be a heparan sulfate binding protein. Although the precise nature of receptor-ligand interactions and down-stream signaling is still being elucidated, it is well known RAGE modulates important pro-inflammatory responses [5]. Our past studies suggest that heparin sulfate directly interacts with RAGE and promotes RAGE oligomerization and signaling. By identifying the heparan sulfate binding sites on RAGE and the hydrophobic residues important for oligomerization, we were able to characterize the structural details between heparan sulfate/RAGE interactions and RAGE oligomerization. Understanding how heparan sulfate regulates RAGE signaling can lead the development of specific molecules or inhibitors to form a blockade at these important heparan sulfate binding and hydrophobic sites to regulate pro-inflammatory responses in various disease states.

We have examined the heparan sulfate-RAGE interactions by combining biophysical methods and mutagenesis to structurally characterize specific amino acids critical for heparan sulfate binding and oligomerization. RAGE mutants including K39A, K43A-K44A, R104A, K107A and R216A-R218 disrupt binding with heparan sulfate. These mutants also prevent the formation of a stable RAGE tetramer which is required for signaling. RAGE mutants including V35A, V78A-L79A, and F85A-L86A disrupt oligomerization which suggests these hydrophobic residues are located on the oligomeric interface of RAGE. The next experimental approach would be to transfect RAGE mutants with impaired binding to heparan sulfate into endothelial cells to verify the significance of heparan sulfate-RAGE interactions in signaling.

We have shown that heparin derived oligosaccharides (minimum size of a dodecasaccharide) is capable of inducing RAGE oligomerization by forming a tetramer. Our studies demonstrates RAGE ligand binding is unaffected by the heparan sulfate sites and oligomeric interface of RAGE. Although important hydrophobic residues and heparan sulfate binding residues of RAGE have been identified to regulate RAGE oligomerization, the organization of the RAGE-oligosaccharide complex would still need to be characterized. A future study would be to construct a structural model of the tetrameric RAGE V-C1

domain (Supplementary Figure 1). Using small-angle X-ray scattering (SAXS), data can be collected in solution and a crystal structure of the monomeric VC1 domain with oligosaccharide can be developed.

Various methods to block RAGE signaling have been explored. Beyond the reported benefits of sRAGE acting as a competitive inhibitor of receptor-ligand binding, pro-inflammatory pathology can be also inhibited by direct receptor blockade with a RAGE V domain-specific antibody [16 17]. Thus, sRAGE and the RAGE V domain peptide can potentially block RAGE dimerization and MAPK/p44 signaling, pathways linked to inflammation. Further blockade of RAGE attenuates NF-κB activation suggesting disruption of the intrinsic RAGE homodimerization and competitive ligand binding can reduce signal activation [5 15].

Based on our studies, we are working to develop monoclonal antibodies (mAbs) that specifically target the heparan sulfate binding sites or oligomerization interface of RAGE. These mAbs will be prepared and tested as antagonists of RAGE signaling in cellular and animal models. These experiments will not only advance our understanding of heparan sulfated-induced receptor oligomerization and its consequences on cell signaling, but will also generate a novel type of pharmacological agent that specifically target the interaction of heparan sulfate with a receptor, a concept that can be applied to other protein targets that interact with heparan sulfate.

FIGURES AND TABLES

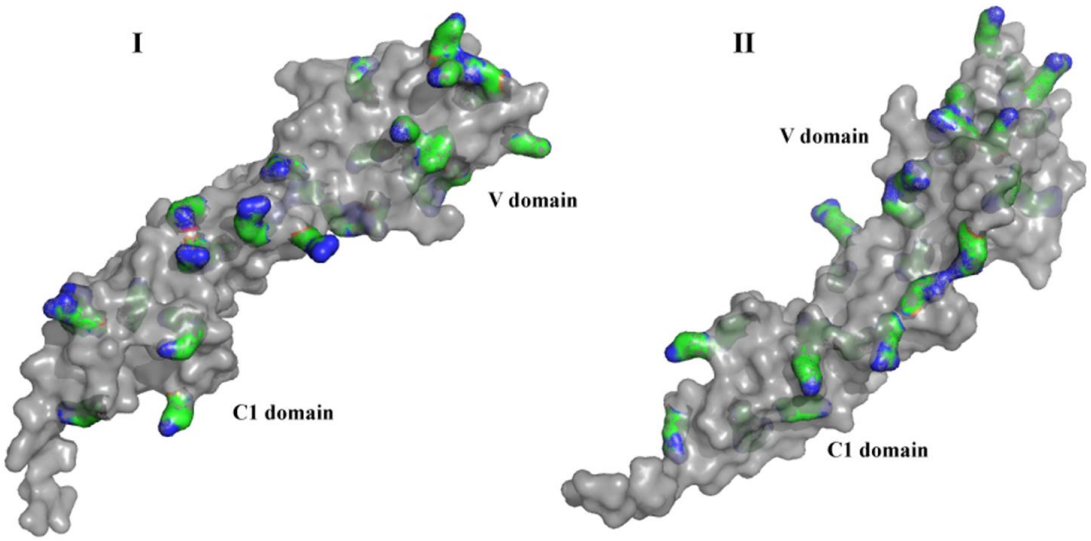


Figure 1: Surface Representation of RAGE VC1 domain

Visual diagram of RAGE VC1 domain (PDB: 3CJJ). Side chains of all lysines and arginines are shown in green (nitrogen in blue). The two potential binding surfaces are approximately in a 135˚ angle with respect to each other along the long axis of V-C1 domain.

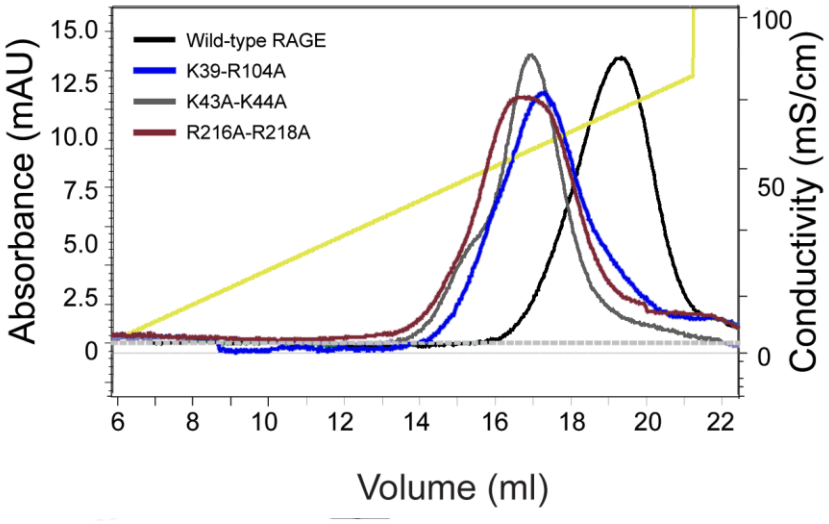


Figure 2: RAGE basic residue mutations affect heparin binding

Wild-type and RAGE basic residue mutants (200 µg) were subjected to heparin sepharose chromatography using a HiTrap heparin high performance column. Bound RAGE protein was eluted with a salt gradient. The conductivity trace is shown as the yellow line.

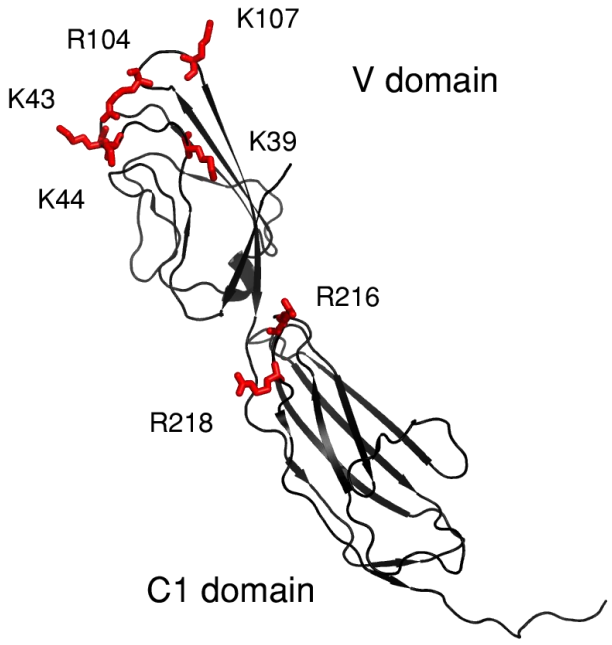


Figure 3: Heparin binding sites on RAGE

Cartoon representation of RAGE VC1 domain (PDB: 3CJJ) with identified heparan sulfate binding residues shown in red.

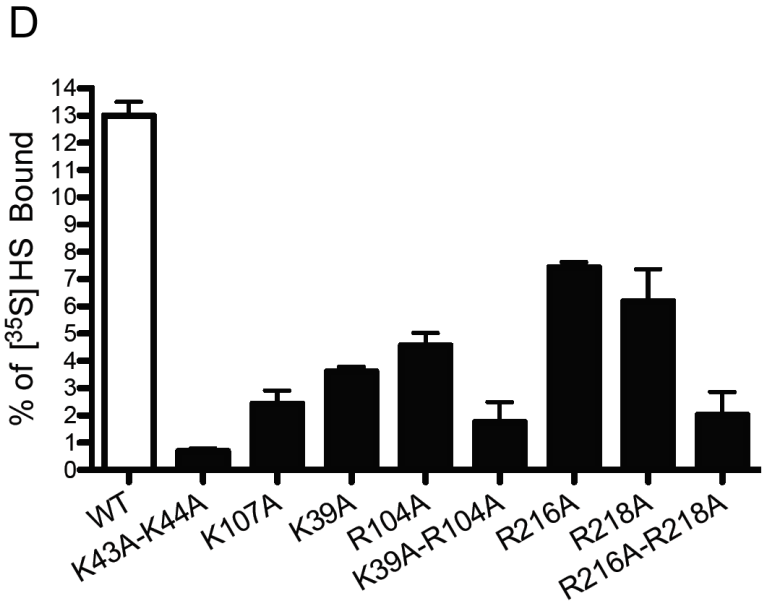


Figure 4: RAGE basic residues mutations also affect heparan sulfate

Binding of wild-type and various mutant RAGE (1 µg) to ^{35}S-labeled endothelial heparan sulfate was determined by a filter binding assay. (n=3, *error bar* represents S.E.) *mAU,* milliabsorbance units.

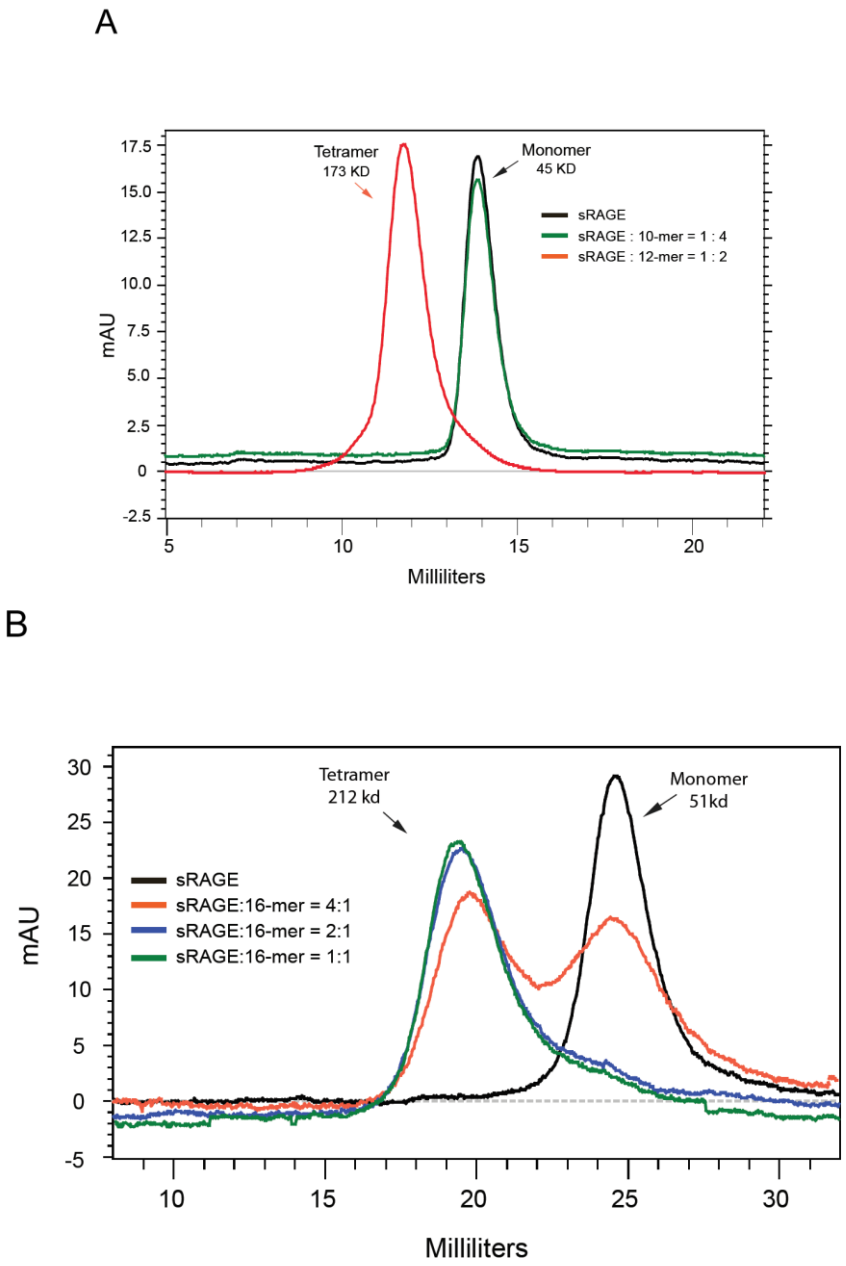


Figure 5: Heparin-derived oligosaccharides induce tetramerization of sRAGE

(A) sRAGE at 30 µM was incubated with various oligosaccharides at indicated molar ratio for overnight at 4 °C. The mixtures were resolved by Superdex 200 (10/300 mm) gel filtration column. The apparent MW was calculated based on protein standards. The purified sRAGE/12-mer tetramer was reapplied onto the column after one week storage.

(B) A gel filtration chromatography depicting the possible stoichiometry of 4 RAGE monomers with 2 heparin oligosaccharides. sRAGE at 30 µM was incubated with hexadecasaccharides (16-mer) at the indicated molar ratio for overnight at 4 °C. The mixtures were resolved by Superdex 200 (10/300 mm) gel filtration column. The apparent MW was calculated based on protein standards.

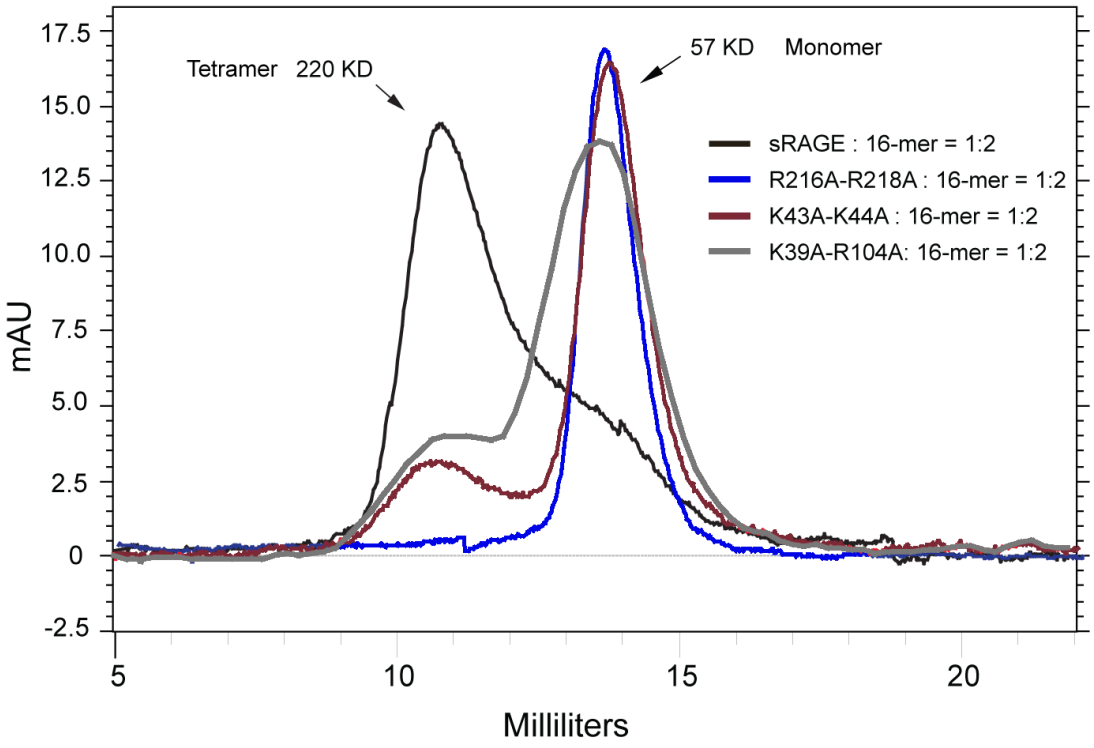


Figure 6: RAGE residues critical for heparan sulfate binding affect RAGE tetramerization

Wild type sRAGE and mutants were incubated with oligosaccharides and mixtures were resolved on a Superdex 200 (10/300 mm) gel filtration column.

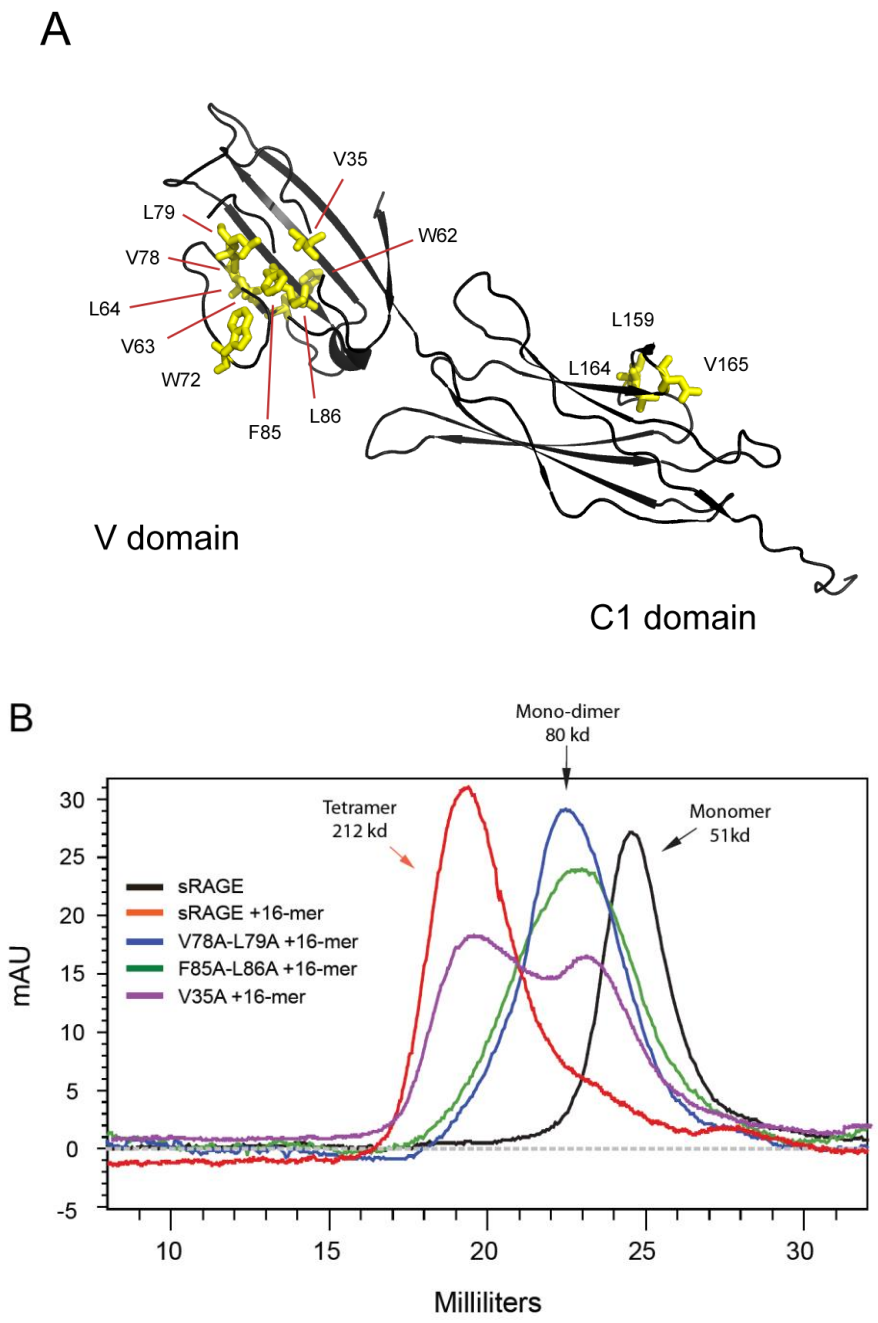


Figure 7: A patch of hydrophobic residues mediates RAGE oligomerization

(A) Cartoon representation of RAGE VC1 domain (*PDB: 3CJJ*) with selected hydrophobic residues for site-directed mutagenesis. Ten residues, located in two hydrophobic patch regions, were mutated to alanine to screen for involvement in oligomerization (shown in yellow).

(B) Mutations of residues belonging to a hydrophobic patch of sRAGE were incubated with heparin-derived oligosaccharide 16-mer) at a 1:1 molar ratio. Mixtures were resolved on a Superdex 200 (10/480 mm) gel filtration column.

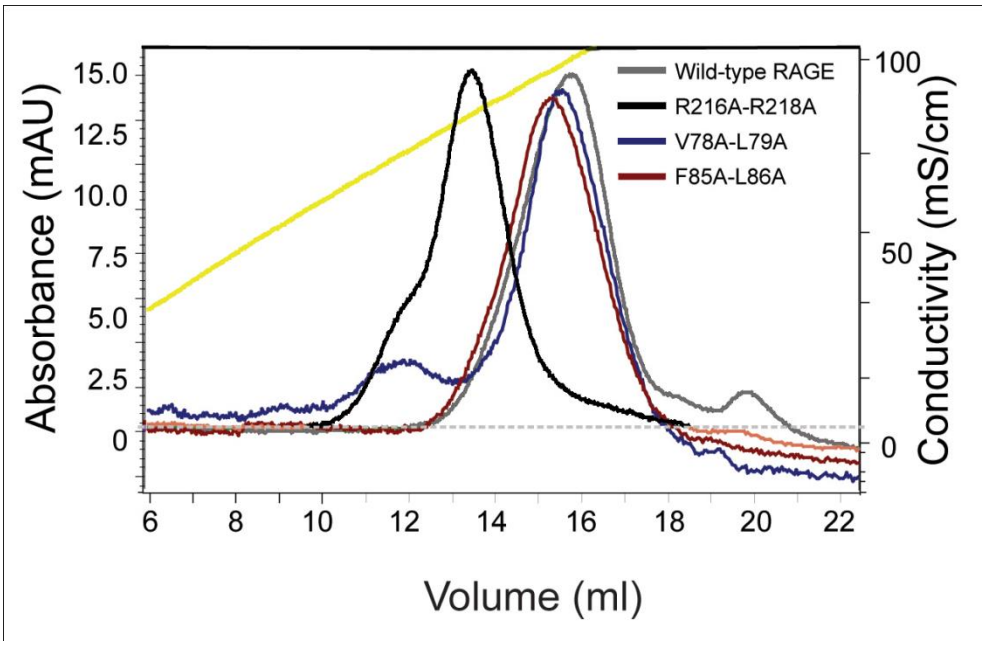


Figure 8: RAGE hydrophobic residue mutations affect heparin binding

Wild-type and RAGE hydrophobic patch mutants (200 µg) were subjected to heparin sepharose chromatography using a HiTrap heparin high performance column. Bound RAGE protein was eluted with a salt gradient. The conductivity trace is shown as the yellow line.

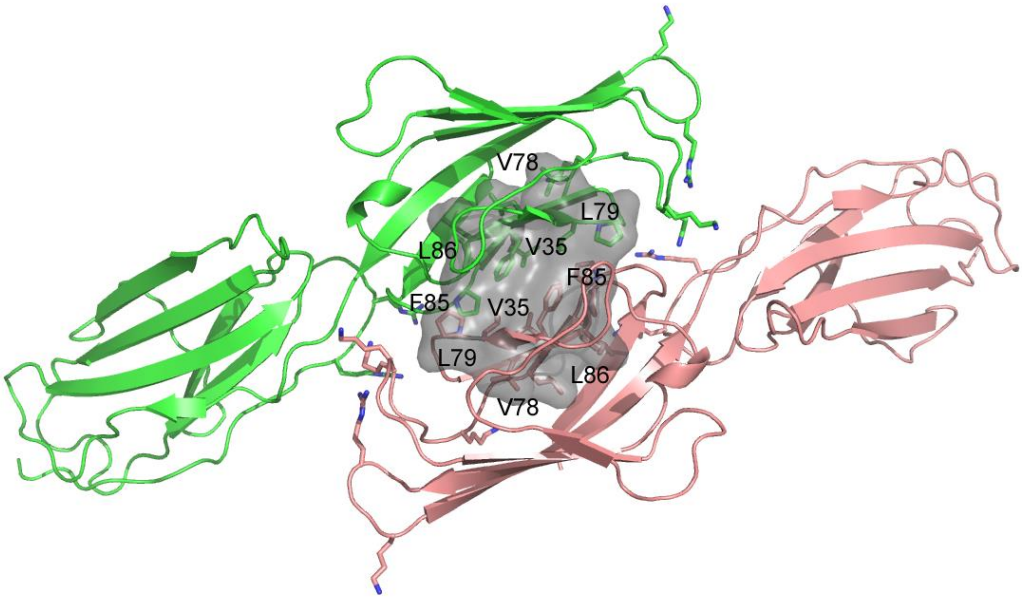


Figure 9: RAGE Dimeric Interface

Cartoon representation of dimeric interface of RAGE VC1 domain. Only two monomers (green and salmon) are shown and the identified hydrophobic dimeric interface is shown as a gray molecular surface. Hydrophobic residues and selected heparan sulfate binding residues are shown in stick.

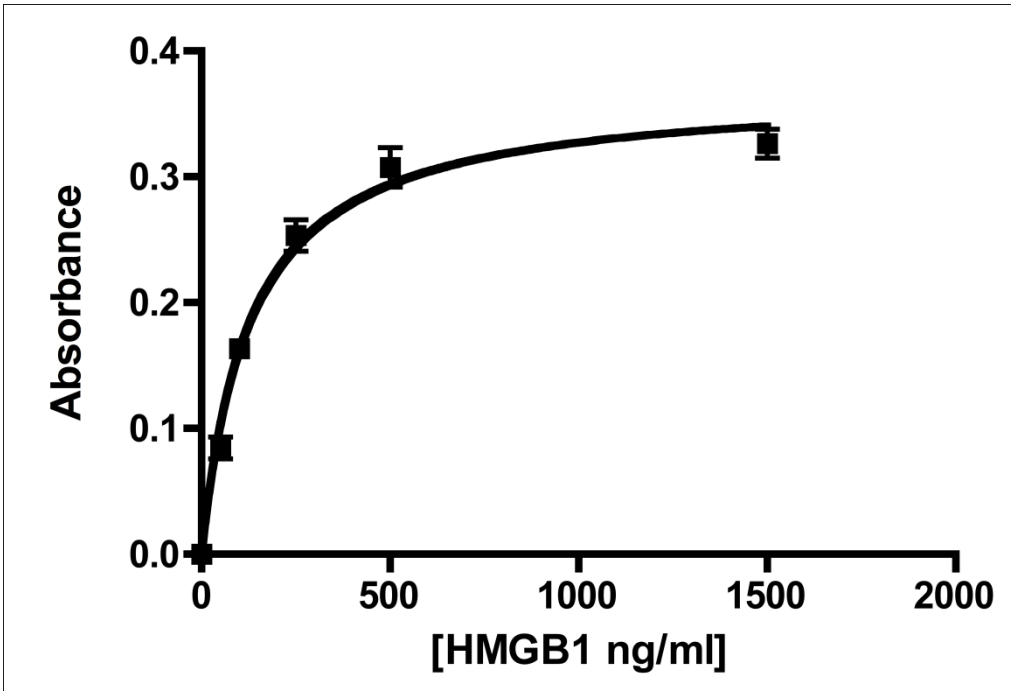
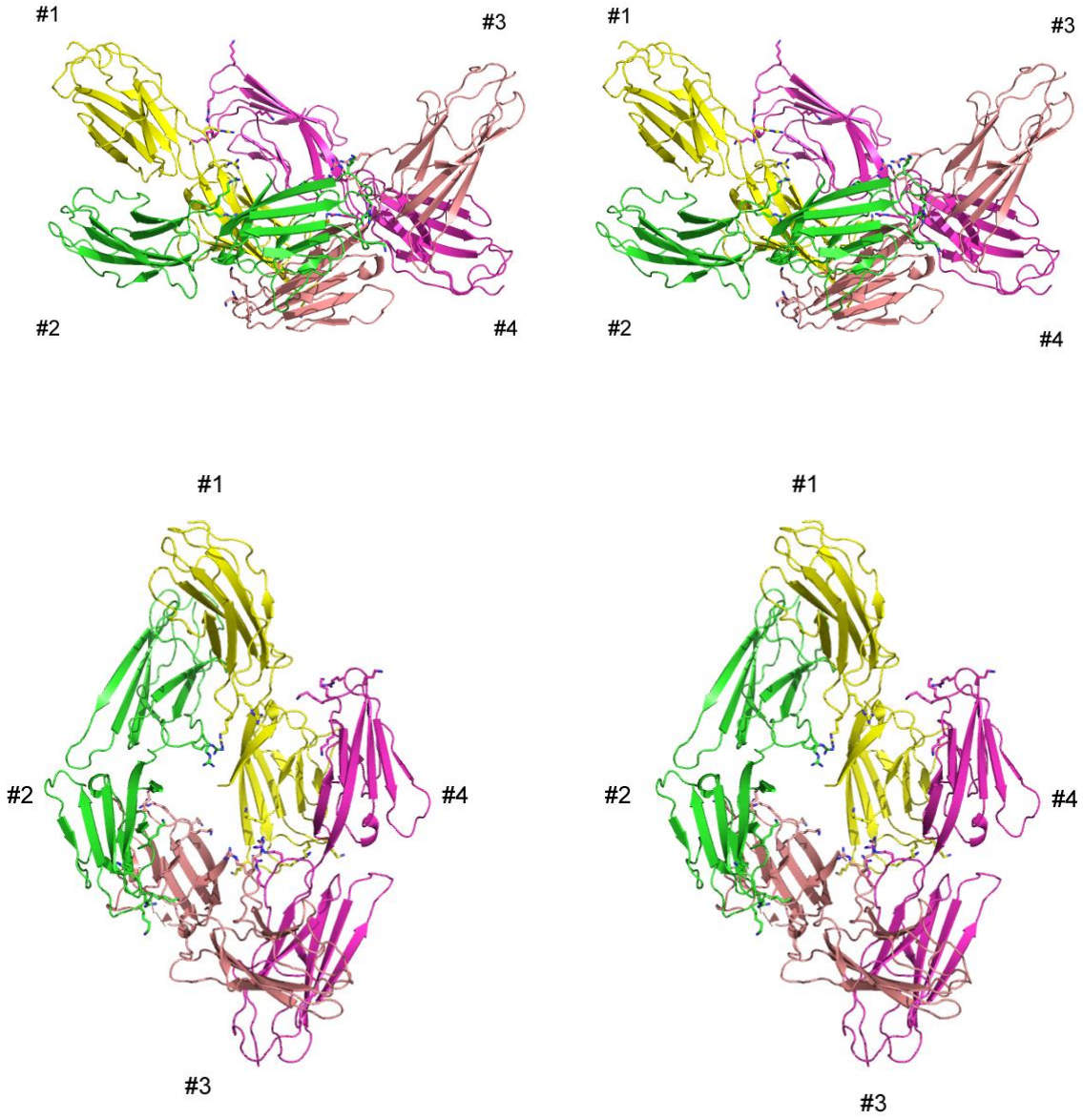


Figure 10: Ligand binding unaffected by heparan sulfate binding sites and oligomeric interface of RAGE

Based on a ligand binding test, wild type sRAGE and mutants were immobilized on ELISA plates and incubated with the indicated concentration of biotin-HMGB1. Bound HMGB1 was measured with streptavidin-HRP. A saturated binding curve was generated through Prism based on one site binding. HMGB1 served as the ligand binding to immobilized sRAGE. K_d values indicate binding affinity for HMGB1 to be 10 nm.



Supplementary Figure 1: Model of tetrameric RAGE VC1 domain

Cartoon representation of VC1 tetramer model based on crystal structure of VC1 monomer. The four monomers (#1-4) are colored in gold, green, salmon, and magenta, respectively. Heparan sulfate binding sites are marked as sticks.

Table 1: List of RAGE basic residue mutations affecting heparin and heparan sulfate binding.

Twenty two conserved lysine and arginine residues of RAGE VC1 domain were mutated to alanine to screen for involvement in binding to heparin Sepharose. Bolded residues indicate a decrease in its salt elution profile with heparin Sepharose.

Mutants	Salt Concentration	Mutants	Salt Concentration
WT RAGE	650 mM	RAGE VC1	680 mM
R29A	650 mM	K52A	630 mM
K43A-K44A	**542 mM**	R57A	635 mM
K39A	**595 mM**	K62A	640 mM
R104A	**585 mM**	R98A	635 mM
K39A-R104A	**550 mM**	**K107A**	**595 mM**
K123A	635 mM	K110A	630 mM
K162A	650 mM	K140A	650 mM
K169A	640 mM	R178A-R179A	650 mM
R216A	**590 mM**	R77A	650 mM
R218A	**600 mM**	R114A	650 mM
R216A-R218A	**570 mM**	R116A	630 mM

Table 2: List of RAGE hydrophobic residue mutations tested in ligand binding assay tests

Select RAGE hydrophobic and heparan sulfate binding residues were subjected to a HMGB1 binding assay test.

Biotin-HMGB1 + sRAGE	Bmax (OD)	K_D (nM)
WT RAGE VC1	0.57 $\pm$ 0.02	10 $\pm$ 0.02
K39A	0.49 $\pm$ 0.01	8.33 $\pm$ 0.55
R104A	0.61 $\pm$ 0.02	9.95 $\pm$ 1.04
K43A-K44A	0.51 $\pm$ 0.01	10.35 $\pm$ 0.85
V78A-L79A	0.45 $\pm$ 0.01	11.2 $\pm$ 1.07
F85A-L86A	0.54 $\pm$ 0.01	12.25 $\pm$ 0.96
K107A	0.54 $\pm$ 0.02	13.11 $\pm$ 1.4
R216A-R218A	0.57 $\pm$ 0.01	9.33 $\pm$ 0.96
V35A	0.34 + 0.01	8.3 $\pm$ 0.78

REFERENCES

1. Bucciarelli, L. G.; Wendt, T.; Rong, L.; Lalla, E.; Hofmann, M. A.; Goova, M. T.; Taguchi, A.; Yan, S. F.; Yan, S. D.; Stern, D. M. RAGE is a multiligand receptor of the immunoglobulin superfamily: implications for homeostasis and chronic disease. *Cell Mol Life Sci* **2002,** *59* (7), 1117-1128.

2. Ramasamy, R.; Yan, S. F.; Herold, K.; Clynes, R.; Schmidt, A. M. Receptor for advanced glycation end products: fundamental roles in the inflammatory response: winding the way to the pathogenesis of endothelial dysfunction and atherosclerosis. *Ann N Y Acad Sci* **2008,** No. 1126, 7-13.

3. Chavakis, T.; Bierhaus, A.; Nawroth, P. P. RAGE (receptor for advanced glycation end products): a central player in the inflammatory response. *Microbes Infect* **2004,** *6* (13), 1219-1225.

4. Yan, S. F.; Ramasamy, R.; Schmidt, A. M. The RAGE axis: a fundamental mechanism signaling danger to the vulnerable vasculature. *Circ Res* **2010,** *106* (5), 842-853.

5. Schmidt, A. M.; Yan, S. D.; Yan, S. F.; Stern, D. M. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *J Clin Invest.* **2001,** *108* (7), 949-955.

6. Raucci, A.; Cugusi, S.; Antonelli, A.; Barabino, S. M.; Monti, L.; Bierhaus, A.; Reiss, K.; Saftig, P.; Bianchi, M. E. 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). *FASFEB J* **2008,** *22* (10), 3716-3727.

7. Koyama, H.; Yamamoto, H.; Nishizawa, Y. RAGE and Soluble RAGE: Potential Therapeutic Targets for Cardiovascular Diseases. *Mol Med.* **2007,** *13* (11-12), 626-635.

8. Xu, D.; Young, J.; Song, D.; Esko, J. D. Heparan sulfate is essential for high mobility group protein 1 (HMGB1) signaling by the receptor for advanced glycation end products (RAGE). *J Biol Chem* **2011,** *286* (48), 41736-41744.

9. Esko, J.; Lindahl, U. Molecular Diversity of heparan sulfate. *J. Clin. Invest* **2001,** *108* (2), 169-173.

10. Ornitz, D. M. FGFs, heparan sulfate and FGFRs: complex. *Bioessays* **2000,** *22* (2), 108-112.

11. Spivak-Kroizman, T.; Lemmon, M. A.; Dikic, I.; Ladbury, J. E.; Pinchasi, D.; Huang, J.; Jaye, M.; Crumley, G.; Schlessinger, J.; Lax, I. Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. *Cell* **1994,** *79* (6), 1015-1024.

12. Lemmon, M. A.; Schlessinger, J. Regulation of signal transduction and signal diversity by receptor oligomerization. *Trends Biochem Sci.* **2002,** *19* (11), 459-463.

13. Park, H.; Boyington, J.; Adsit, F. The 1.5 Å crystal structure of human receptor for advanced glycation endproducts (RAGE) ectodomains reveals unique features determining ligand binding. *J Biol Chem* **2010,** *285* (52), 40762-40770.

14. Ostendrop, T.; Leclerc, E.; Galichet, A.; Koch, M.; Demling, N.; Weigle, B.; Heizmann, C. W.; Kroneck, P.; Fritz, G. Structural and functional insights into RAGE activation by multimeric S100B. *EMBO J* **2007,** *26* (16), 3868-3878.

15. Zong, H.; Madden, A.; Ward, M.; Mooney, M. H.; Elliott, C. T.; Alan, S. W. Homodimerization is essential for the receptor for advanced glycation end products (RAGE)-mediated signal transduction. *J*

Biol Chem **2010,** *285* (30), 23137-23146.

16. Yan, S. F.; Ramasamy, R.; Schmidt, A. M. Soluble RAGE: Therapy & Biomarker in Unraveling the RAGE Axis in Chronic Disease and Aging. *Biochem Parhmacol* **2010,** *79* (10), 1379-1386.

17. Xie, J.; Reverdatto, S.; Frolov, A.; Hoffmann, R.; Burz, D. S.; Shekhtman, A. Structural Basis for Pattern Recognition by the Receptor for Advanced Glycation End Products (RAGE). *J Biol Chem* **2008,** *283* (40), 27255-27269.

18. Rauvala, H.; Rouhiainen, A. RAGE as a receptor of HMGB1 (Amphoterin): roles in health and disease. *Curr Mol Med* **2007,** *7* (8), 725-734.