Supporting Information

for

Stable RAGE-Heparan Sulfate Complexes are Essential for Signal Transduction

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Supplemental Methods

Recombinant protein expression. Recombinant sRAGE or RAGE V-C1 domain was produced in E. coli. The complete open reading frame of human sRAGE (23-326), human RAGE V-C1 domain (23-232), or mouse RAGE V-C1 domain (23-231) was cloned into pET21b (Novagen) using Not I and Xba I sites. The expression was carried out as described in Origami-B cells (Novagen) carrying the pGro7 (Takara, Japan) plasmid expressing chaperonin proteins GroEL and GroES of E. coli. Purification was carried out using HiTrap SP cation exchange column at pH7.8 (HEPES buffer), followed by SEC on a Superdex 200 column in 20 mM Tris, 200 mM NaCl, pH 8.1(GE healthcare). In all cases, the purity was greater than 98% as determined by SDS-PAGE and Coomassie staining.

Site-directed mutagenesis. RAGE mutants were prepared using pET21b harboring either human sRAGE or mouse RAGE V-C1 domain as the template and a method modified from Agilent Quick-change site-directed mutagenesis kit. Mutants were confirmed by sequencing, and recombinant protein was expressed and purified as described for wild-type RAGE.

SEC-Multiple angle light scattering (MALS). sRAGE/dodecasaccharide complexes were resolved on a SEC HPLC column (7.8 x 300mm, Wyatt Technology WTC-03S5) and the eluate was passed in-line to a miniDAWN TREOS MALS detector followed by a Optilab T-rEX refractive index detector (Wyatt Technology). The Wyatt software ASTRA was used to analyze all the collected data.

Ligand binding ELISA. 200 ng of RAGE mutants were immobilized onto 96-well high-binding ELISA plate. Plates were blocked with 5% BSA in PBS and incubated with various concentrations of biotinylated HMGB1 or S100b for 1 hour at room temperature. Bound ligands were quantitated with streptavidin-HRP (Jackson Immunology) followed by the addition of HRP substrate (Thermo Scientific). B_{max} and K_d were calculated by fitting the binding data to a single-site binding model in Prism 5.0.

To assess the effect of anti-RAGE antibodies on ligand binding to RAGE, HMGB1 or S100b (200 ng) were immobilized and the plate blocked as described above. Biotinylated-mouse V-C1 domain (200 ng/ml) was pre-incubated with rabbit IgG, polyclonal or monoclonal anti-RAGE (all at 5 μg/ml) for 30 minutes at room temperature before added into the plate. Quantification of binding was performed as described above. The percentage of antibody inhibition was calculated by comparing the absorbance obtained at the presence of the antibodies to a binding curve of V-C1 (using 20ng to 1μg/ml) to the respective ligands.

SAXS data analysis. The data were first analyzed in a Guinier plot using PRIMUS (3.1). The pair-distribution function P(r) was calculated using GNOM (4.6) [1,2]. The molecular weight estimation of the complexes based on SAXS scattering curve was performed using the program SaxsMoW (1.0) [3]. The final scatting curve used for modeling was prepared by merging SAXS experimental data (low-concentration data for small q and high-concentration data for high q). For ab initio modeling of the molecular envelope, 20 GASBOR runs (P3 symmetry) was performed, from which an averaged model was calculated using the program DAMAVER (3.2) [4,5]. SUPCOM (1.3) was used for superimposing the crystal structure to the GASBOR models. [6]. The theoretical

scattering curve of the crystal structure was calculated and fitted to the experimental curve by the program FoXS [7].

Reference

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Supplemental Table 1. Salt concentration for elution of RAGE mutants from heparin-Sepharose

Mutant	Peak NaCl
WT His-sRAGE	650 mM
R29A	655 mM
K39A	**600 mM**
K43A-K44A	**540 mM**
R104A	**585 mM**
K123A	630 mM
K162A	650 mM
K169A	640 mM
K52A	630 mM
R57A	635 mM
K62A	645 mM
R98A	630 mM
K107A	**595 mM**
K110A	630 mM
K140A	650 mM
R178A-R179A	660 mM
R77A	660 mM
R114A	665 mM
R116A	630 mM
R216A	**590 mM**
R218A	**600 mM**
R216A-R218A	**575 mM**
K39A-R104A	**550 mM**
V35A	650 mM
V78A-L79A	635 mM
F85A-L86A	645 mM

Supplemental Table 2. Binding parameters of RAGE mutants to ligands.

Mutant	HMGB1		S100b	
	[1]B_{max}	[2]K_d	B_{max}	K_d
WT sRAGE	0.57 ± 0.02	10.0 ± 0.9	0.24 ± 0.03	83 ± 27
K39A	0.49 ± 0.01	8.3 ± 0.5	0.24 ± 0.03	88 ± 26
K43A-K44A	0.51 ± 0.01	10.3 ± 0.8	0.26 ± 0.04	77 ± 30
R104A	0.61 ± 0.02	10.0 ± 1.0	0.25 ± 0.03	90 ± 20
K107A	0.54 ± 0.02	13.1 ± 1.4	0.37 ± 0.04	63 ± 23
R216A-R218A	0.57 ± 0.01	9.3 ± 0.9	0.28 ± 0.04	85 ± 20
V35A	0.54 ± 0.02	8.3 ± 0.8	0.39 ± 0.02	72 ± 10
V78A-L79A	0.45 ± 0.01	11.2 ± 1.1	0.29 ± 0.04	62 ± 27
F85A-L86A	0.54 ± 0.01	12.2 ± 0.9	0.33 ± 0.05	78 ± 34

[1] B_{max} is in absorbance unit.
[2] K_d is in nM.

Supplemental Table 3. Crystallographic Data Statistics

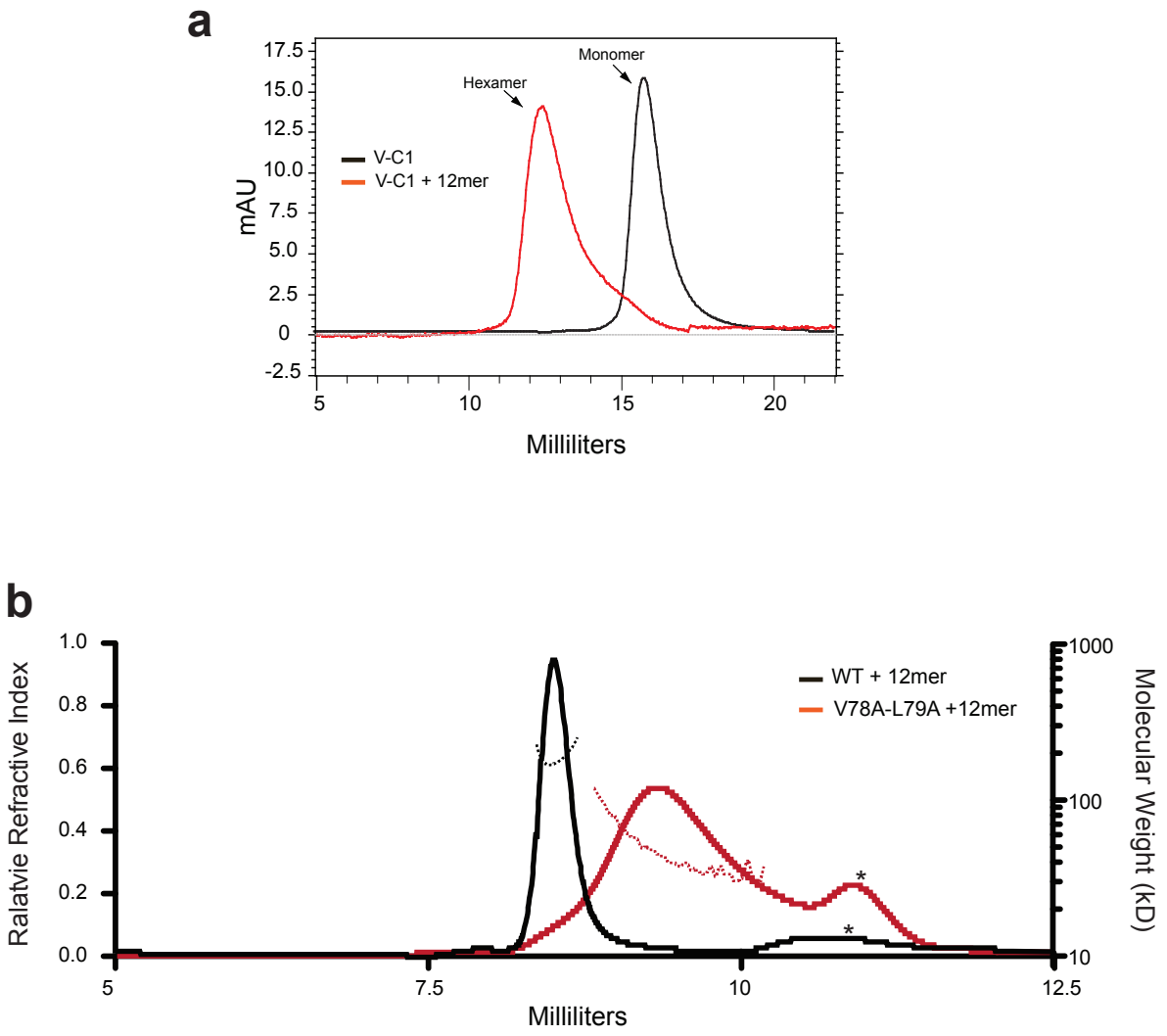
Crystallographic Data Statistics	
unit cell	a= b= c=115.81 Å; $\alpha=\beta=\gamma=90°$
Space Group	$P4_132$
Resolution (Å)	50.0 – 3.5
# of observations	48,106
Unique reflections	3672
Rsym(%)(last shell)[1]	10.7 (70.2)
I/σI (last shell)	8.1 (4.0)
Mosaicity range	0.52-0.66
Completeness (%) (last shell)	99.5 (100)
Refinement statistics	
Rcryst(%)[2]	25.8
Rfree(%)[3]	33.6
# of waters	0
Overall Mean B value (Å)	121.5
r.m.s. deviation from ideal values	
bond length (Å)	0.016
bond angle (°)	1.76
dihedral angle (°)	13.54
Ramachandran Statistics[4]	
residues in:	
favored (98%) regions (%)	93.75
allowed (>99.8%) regions (%)	99.48

1) Rsym = $\sum (| I_i - < I>|)/ \sum(I_i)$ where I_i is the intensity of the ith observation and <I> is the mean intensity of the reflection.
2) Rcryst = $\sum|| Fo| - | Fc ||/ \sum| Fo|$ calculated from working data set.
3) Rfree was calculated from 5% of data randomly chosen not to be included in refinement.
4) Ramachandran results were determined by MolProbity.

Supplemental Table 4. SAXS Data Characterizations.

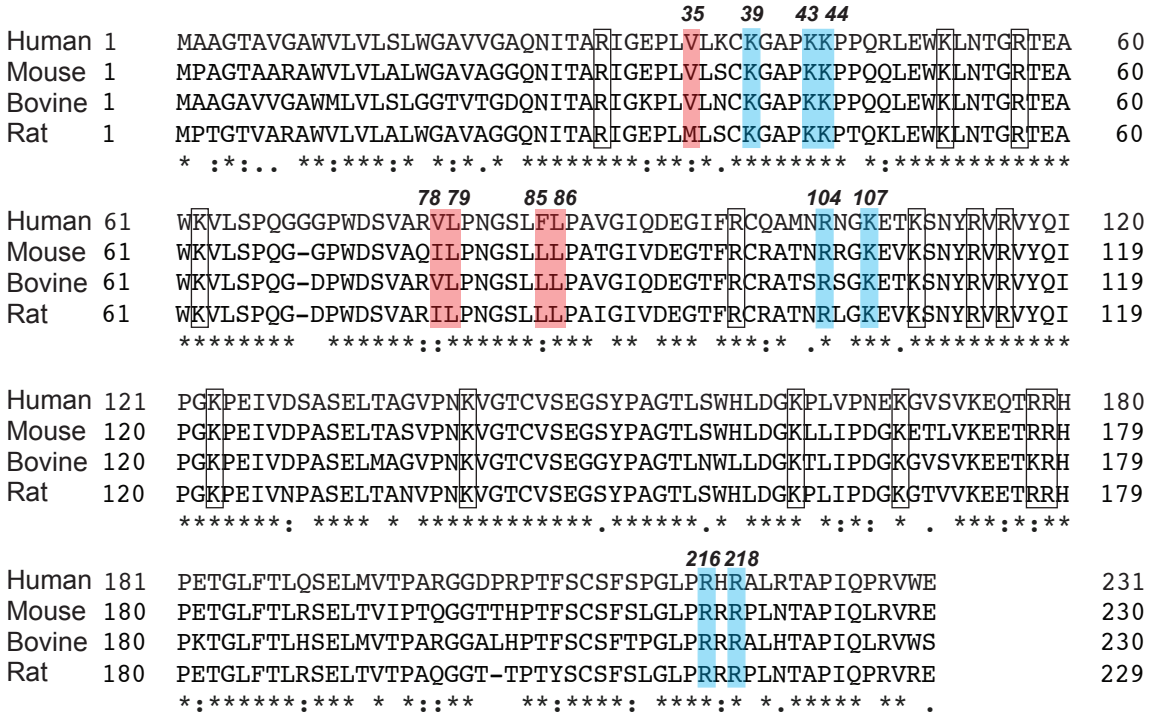
	R_g (Å) Guinier	**R_g (Å)** P(r) function	**D_{max} (Å)** P(r) function	**MW(kDa)** SaxsMoW	**MW(kDa)** Theoretical
sRAGE Hexamer	51.6	51.7	165	216	202
VC1 Hexamer	38.7	38.8	130	134	142
VC1 monomer	26.4	26.6	84	24.4	23

Supplemental Figure 1



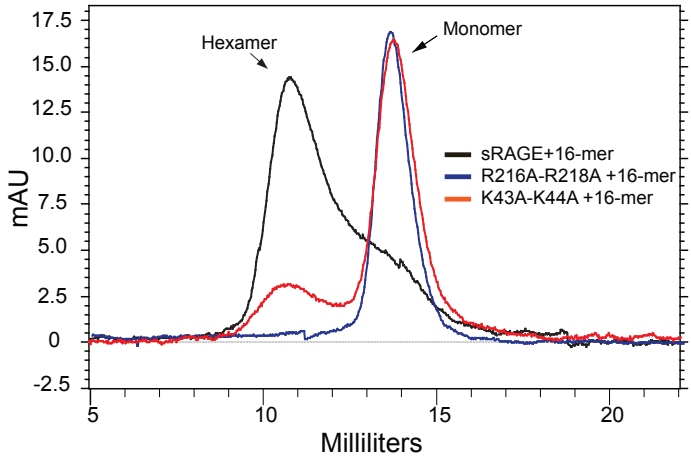
Supplemental Figure 1. (**a**) Dodecasaccharide induces a stable hexamer of RAGE V-C1 domain. Mouse RAGE V-C1 domain was incubated with dodecasaccharide at room temperature overnight and resolved on a Superdex 200 (10/300 mm) gel filtration column. (**b**) SEC-MALS analysis of wild-type sRAGE/dodecasaccharide complex and V78A-L79A/dodecasaccharide complex. Relative refractive index traces are shown by bold lines and the molecular weights measurements are shown by dotted lines. Asterisks indicate excessive oligosacchride. Samples are resolved on a HPLC size exclusion column (7.8 x 300 mm) with a guard column attached.

Supplemental Figure 2



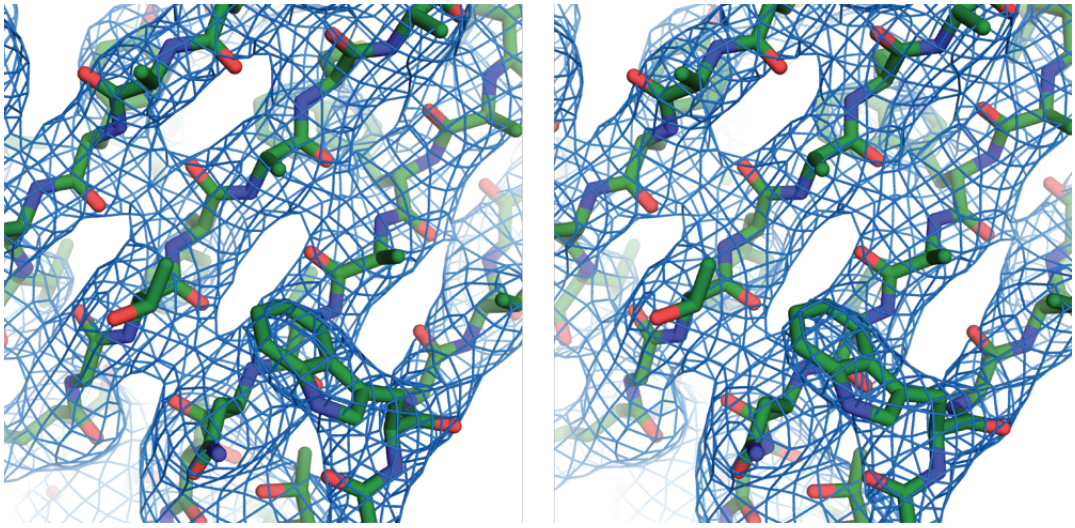
Supplemental Figure 2. Multiple-species sequence alignment of RAGE V-C1 domains. Basic residues that were mutated but had no effect on heparin binding are boxed in black. Heparan sulfate–binding residues are shaded blue, and mutated hydrophobic residues are shaded red.

Supplemental Figure 3



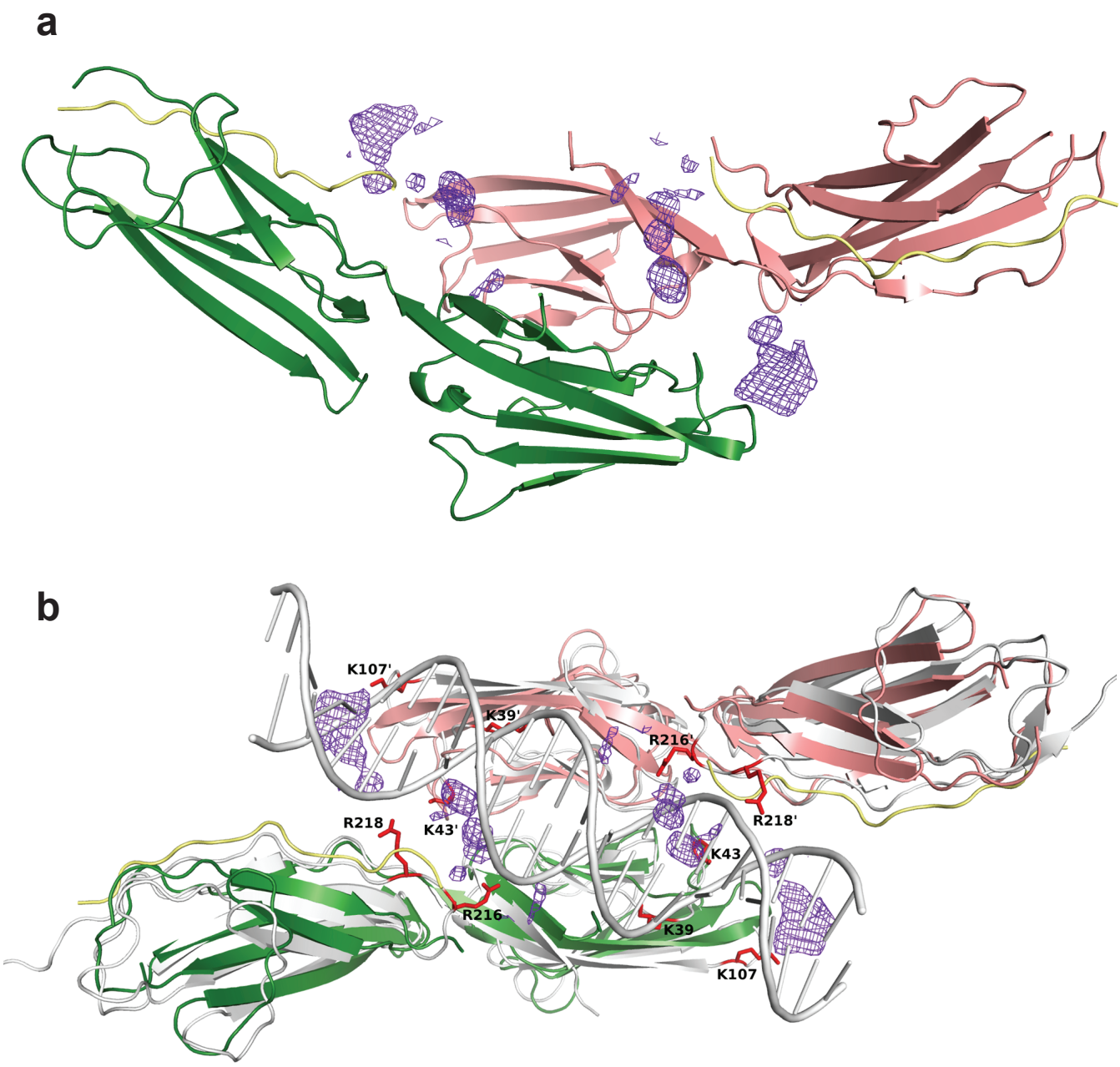
Supplemental Figure 3. RAGE mutants defective in heparan sulfate binding were unable to form hexamer. sRAGE mutants were incubated with oligosaccharides and the mixtures were resolved on a Superdex 200 (10/300mm) gel filtration column.

Supplemental Figure 4



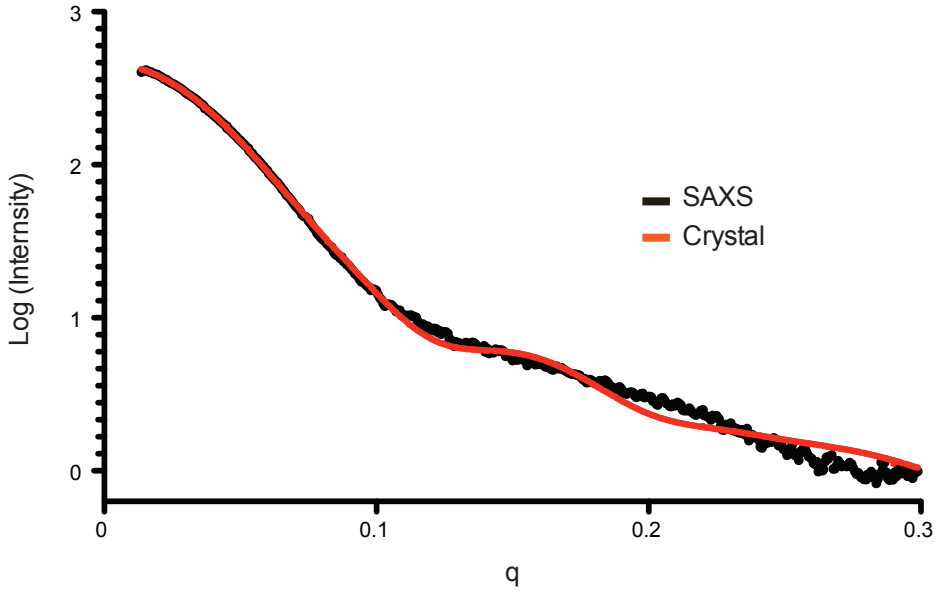
Supplemental Figure 4. 2Fo-Fc electron density map of mRAGE V-C1. 2Fo-Fc electron density (blue) from the V domain of the 3.5 Å crystal structure of mRAGE V-C1 (green) contoured at 1 σ.

Supplemental Figure 5



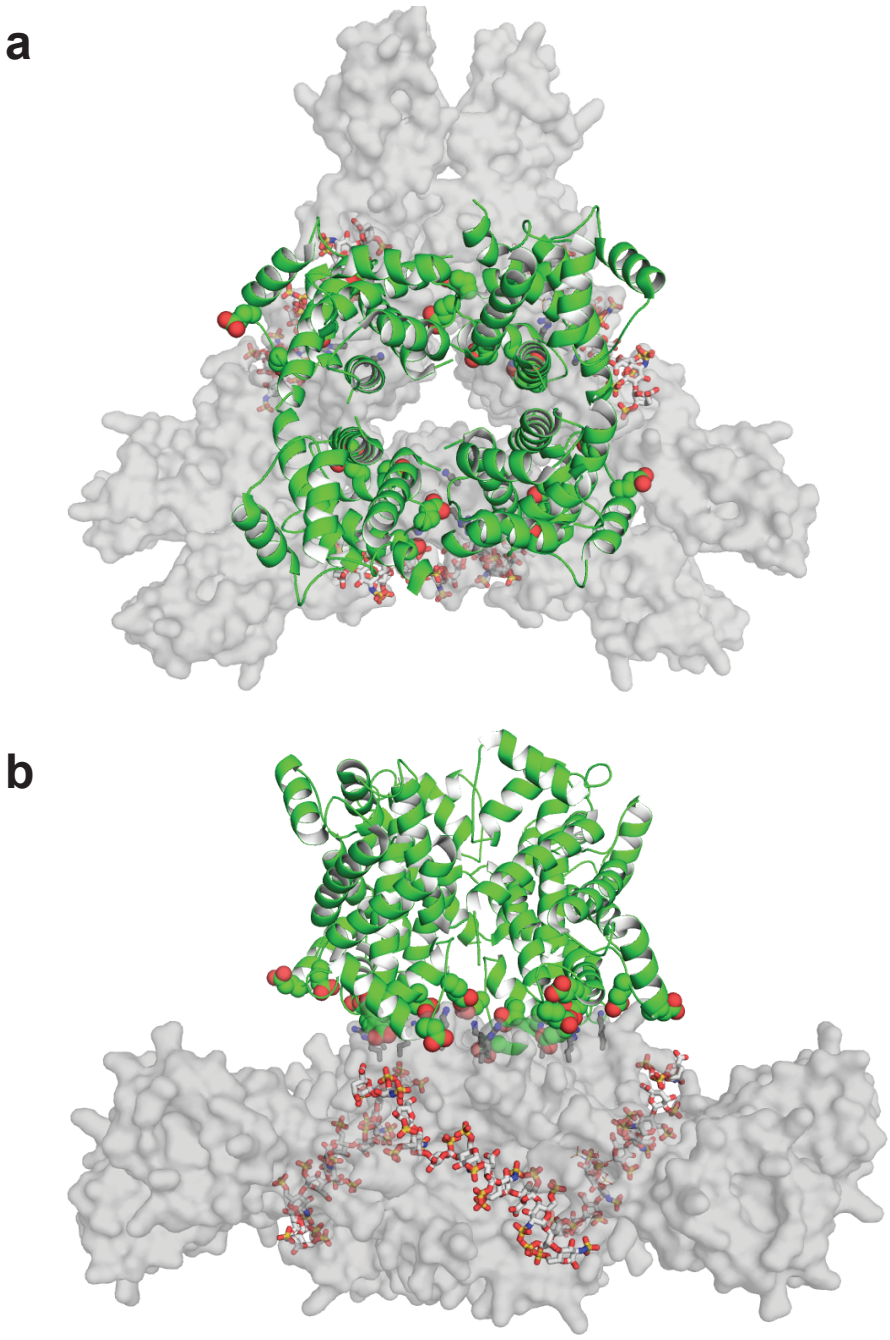
Supplemental Figure 5. (**a**) Difference density at the interface of the mRAGE V-C1 dimer. Crystallographic dimer of the mRAGE V-C1 construct. Unmodeled Fo-Fc density (violet) contoured at 2.5σ is found along the interface of the two mRAGE monomers (green and salmon). The "swapped" C-terminal tails from these monomers have been removed from the figure and those from neighboring molecules in the crystal are shown in yellow. (**b**) PDB coordinates 3S59 with bound DNA (grey) was superimposed with the mRAGE V-C1 dimer. The position of the difference density is consistent with the DNA binding position to hRAGE V-C1 in 3S59. Residues that make contact with the DNA and are near the difference density are shown in red.

Supplemental Figure 6



Supplementary Figure 6. Comparison of experimental SAXS curve to theoretical SAXS cruve calculated from hexameric RAGE V-C1 crystal structure. Calculation of the theoretical scattering curve from the crystal structure (with three modeled dodecasacchrides) was performed by the program FoXS, and the agreement between the curves has a Chi = 2.53.

Supplemental Figure 7



Supplemental Figure 7. Proposed binding mode of S100b octamer to mRAGE V-C1 hexamer. Top view (**a**) and side view (**b**) with hexameric V-C1 domains shown in grey surface, dodecasaccharides shown in sticks with white backbone, K52, R98 and K110 of V domain shown in sticks with dark grey backbone, octameric S100b (PDB-id: 2H61) shown in green cartoon and acidic residues of S100b (E21, E31, E34, E46 and E49) shown in sphere. The lower panel depicts the complex oriented relatively to the plasma membrane (not shown) below the complex.