The receptor for advanced glycation end products (RAGE) is a multiligand receptor that is implicated in the pathogenesis of various diseases, including diabetic complications, neurodegenerative disorders, and inflammatory responses. The ability of RAGE to recognize advanced glycation end products (AGEs) formed by nonenzymatic glycoxidation of cellular proteins places RAGE in the category of pattern recognition receptors. The structural mechanism of AGE recognition was an enigma due to the diversity of chemical structures found in AGE-modified proteins. Here, using NMR spectroscopy we showed that the immunoglobulin V-type domain of RAGE is responsible for recognizing various classes of AGEs. Three distinct surfaces of the V domain were identified to mediate AGE-V domain interactions. They are located in the positively charged areas of the V domain. The first interaction surface consists of strand C and loop CC, the second interaction surface consists of strand C', strand F, and loop FG, and the third interaction surface consists of strand A' and loop EF. The secondary structure elements of the interaction surfaces exhibit significant flexibility on the ms-μs time scale. Despite highly specific AGE-V domain interactions, the binding affinity of AGEs for an isolated V domain is low, ~10 μM. Using in-cell fluorescence resonance energy transfer we show that RAGE is a constitutive oligomer on the plasma membrane. We propose that constitutive oligomerization of RAGE is responsible for recognizing patterns of AGE-modified proteins with affinities less than 100 nM.

The receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily that binds a broad repertoire of ligands (1, 2). In humans and mice, RAGE is located within the major histocompatibility class III locus, which encodes members of the innate immune system (3, 4). RAGE consists of three extracellular immunoglobulin-like domains, V, C1, and C2, a transmembrane helix, and a short (42 amino acids) intracellular, negatively charged C-terminal tail, indispensable for RAGE signaling (2).

RAGE is not absolutely required for normal physiology (5). RAGE−/− mice, lacking RAGE, displayed normal macroscopic pathology and histopathology. RAGE−/− mice also exhibited normal initiation and perpetuation of inflammation in models addressing predominantly the adaptive immune response. At the same time RAGE−/− mice were protected from the lethal effects of septic shock, which depends largely on the innate immune response (5, 6). These results suggest a strong link between RAGE and innate immunity.

The first identified ligands of RAGE were products of nonenzymatic glycoxidation termed advanced glycation end products (AGEs) (1, 7). AGEs are a heterogeneous group of structures, including carboxymethyllysine (CML), carboxyethyllysine (CEL), and hydroimidazolone protein adducts that are formed at enhanced rates at sites of inflammation in renal failure, under conditions of hyperglycemia, and under conditions associated with systemic and local oxidant stress (8–10). There is substantial evidence linking AGEs to the development of vascular complications of diabetes, uremia, and aging, with less well established links to non-diabetic vascular disease, Alzheimer disease, and inflammatory disorder (11).

Despite their structural diversity, AGEs bind only to the V domain of RAGE (7). This binding does not accelerate clearance or degradation but, rather, begins a sustained period of cellular activation mediated by receptor-dependent signaling, leading to inflammation. It is proposed that RAGE activation is largely responsible for the pathogenicity of AGEs (2, 12).

In addition to AGEs, other endogenous ligands are implicated in amplifying RAGE-dependent proinflammatory signaling: Cytokine-like mediators of the S100 family (2) and amphoterin (13), a nuclear protein released by necrotic cells. Unlike AGEs and possibly amphoterin, which bind to a single domain, all three extracellular domains of RAGE are involved in binding S100 proteins; S100A12 binds to the C1 domain (14), S100B binds to V and C1 (15), and S100A6 binds to V and C2 (16). These differences in RAGE binding sites may account for the diverse cellular responses caused by S100 proteins.

Two glycosylation sites were identified in RAGE (17). Glycosylation at the V domain site significantly increases RAGE affin-
ity for amphoterin, presumably due to the amphoterin binding affinity for carboxylated glycans. At the same time, de-glycosylation as well as G82S polymorphism in the V domain increases RAGE affinity for specific AGEs (18, 19).

Soluble RAGE (sRAGE), consisting of the extracellular V, C1, and C2 domains, was also found in plasma (20). This protein can scavenge RAGE ligands, thus preventing them from binding to RAGE and/or other surface cell receptors. sRAGE serves as a decoy rather than a selective RAGE blocker and may have wider therapeutic potential than RAGE. sRAGE administered to diabetic mice significantly suppressed accelerated atherosclerosis, which is a hallmark of inflammation (21).

The structural diversity of RAGE ligands and the fact that RAGE recognizes a class of ligands such as AGEs led to the hypothesis that RAGE is a pattern recognition receptor (PRR) (5, 22). The concept of pattern recognition was introduced to describe innate immunity receptors, such as the Toll and mannose receptors, which recognize conserved molecular structures shared by a large group of pathogens (23). Such conserved patterns can be a carbohydrate moiety on the pathogen cell wall or a viral double-stranded RNA. The mannose receptor, one of the most extensively studied PRRs, serves as a paradigm of this class of receptors (24) and has the following features common to all PRRs: 1) a multidomain structure, 2) repeats of structurally similar subunits, 3) the ability to recognize different classes of ligands (proteins, carbohydrate chains, etc.), confined to specialized domains, and 4) in the case of polysaccharide binding, binding affinity of monomeric units is weak and increases with increasing polymer chain length (25).

Features 1 and 3 are present in RAGE. However, RAGE does not have repeats of structurally similar domains, which allows PRRs to recognize patterned ligands. Unlike PRRs of the innate system that predominantly bind exogenous ligands, all known RAGE ligands are endogenous. RAGE binding affinity for AGEs strongly depends on the degree of glycation of the ligand. Paradoxically, free glycated amino acids, CML and CEL, do not strongly depend on the degree of glycation of the ligand. Paradoxically, free glycated amino acids, CML and CEL, do not bind to RAGE, although various AGE-modified proteins bind to RAGE with nanomolar affinity (7).

Here we present a series of experiments that are designed to elucidate the mechanism of RAGE-AGEs binding and show how RAGE functions as a PRR. Using fluorescence resonance transfer (FRET), we showed that expressed RAGE oligomerizes on the plasma membrane of human cells. Using high resolution NMR, we constructed a structural model of the AGE binding V-domain of RAGE. We identified the interaction surfaces used by RAGE to bind different classes of AGEs. We also determined the influence of the G82S mutation of RAGE on its structure and the binding affinity for AGEs. These experiments allowed us to propose a consistent mechanism of RAGE-AGEs structural interactions that can shed light on the complex nature of RAGE signaling in the cell.

**EXPERIMENTAL PROCEDURES**

**Reagents and Chemicals**—Restriction enzymes and Taq polymerase were from New England Biolabs. N*-carboxymethyl)-1-lysine and N*-carboxyethyl)-1-lysine (mixture of two diastereoisomers) were from NeoMPS, Inc. (purity > 95%). All other chemicals were reagent grade or better.

**Cell Culture and Transfection**—HEK293 cells from the American Type Culture Collection were cultured in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%), penicillin (5 μg/ml), and streptomycin (5 μg/ml), all from Invitrogen. Cells were maintained at 5% CO2 and 37°C. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) and Opti-MEM media (Invitrogen) following the manufacturer’s guidelines. Cells were routinely incubated with transfection mixtures overnight, medium was exchanged to modified Eagle's medium without phenol red (Invitrogen), and cells were imaged live within the next 5–30 h.

**Plasmid Construction and cDNAs**—Human RAGE cDNA library clone BC020669 was obtained from Open Biosystems and used as a template for PCR amplifications. Clontech vectors containing genes for cyan fluorescent protein (pCFP-N1) and yellow fluorescent protein (pYFP-N1) were mutated using the QuikChange site-directed mutagenesis II-E kit (Stratagene) to introduce an A206K substitution in both CFP and YFP, which eliminates the tendency of CFP and YFP to dimerize at high concentrations (26). We called these mutants mCFP and mYFP, where “m” denoted monomeric versions of the proteins. hRAGE cDNA was cloned in-frame into modified vectors upstream of the fluorescent protein sequence to obtain hRAGE-mCFP and hRAGE-mYFP constructs. DNA coding for the V domain (amino acids 24–125) and C1C2 domains (amino acids 114–334) were PCR-amplified from cDNA library clone BC020669 (Open Biosystems) using Taq polymerase and oligonucleotides containing flanking 5'-Ndel and 3'-Sall restriction sites. The restriction-digested PCR products were ligated into the Ndel and Sall sites of expression vector pET28a (Novagen), which confers kanamycin resistance. The resulting plasmids pET28V and pET28C12 express cleavable, C-terminal His-tagged proteins. The QuikChange site-directed mutagenesis II-E kit (Stratagene) was used to introduce a G82S substitution into the V domain.

**Confocal Microscopy and FRET Assay**—HEK293 cells were plated on 35-mm dishes with polylysine-coated glass coverslip windows (MatTek) and grown to 90% confluence before transfecting with hRAGE/mCFP (donor) or hRAGE/mYFP (acceptor) plasmids or with both simultaneously at a donor/acceptor ratio of 1:2. Invitrogen recommendations on the cell to DNA ratio for Lipofectamine transfections were used as guidelines. Live imaging of the cells was performed at room temperature in modified Eagle’s medium (w/o phenol red) using a Zeiss LSM 510 META confocal imaging system equipped with a 30-milliwatt argon laser and a x63, 1.4 NA oil immersion objective. Cells displaying similar levels of labeling throughout the plasma membrane were selected for FRET experiments.

FRET was measured by acceptor photobleaching (27, 28). An optical slice about 2-μm through the middle of HEK293 cells expressing both mCFP and mYFP-labeled RAGE was visualized with a confocal microscope, and a small rectangular region of plasma membrane was selected for photobleaching. Time series mode was used to acquire a series of images at discrete wavelengths (λ-stacks). Images in 12 different channels from 464 to 700 nm were recorded simultaneously after excitation at 458 nm (10% laser intensity was used to minimize donor photobleaching). After collecting 2–3 pre-bleach λ-stacks, selected
regions were bleached using a 514-nm laser line (100% intensity, 60 iterations using a 458/514-nm dual dichroic mirror) effectively decreasing YFP fluorescence to 5–15% that of the pre-bleach levels. Several post-bleach images were collected immediately after photobleaching. For linear unmixing of emission spectra in cotransfected cells, reference spectra of CFP and YFP were collected from cells expressing hRAGE-mCFP or hRAGE-mYFP alone after excitation at 458 nm. Unmixing was facilitated by the use of a Zeiss META detector and the linear unmixing function of the LSM 510 META. Quantitatively, FRET was measured as an increase in CFP fluorescence intensity (donor quenching) after YFP (acceptor) photobleaching. The FRET macro in the Zeiss AIM software package was used for calculations, taking into account CFP and YFP background noise in each channel. FRET efficiencies were also calculated in cells expressing hRAGE-mCFP alone and in non-bleached regions of the cell membrane as a control.

Labeling, Expression, and Purification of the V Domain—To uniformly label the V domain of RAGE, pET28V and pET28V-G82S were transformed into *Escherichia coli* strain BL21(DE3) Codon + (Novagen). For U-13C,N labeling, cells were grown at 37 °C in minimal medium (M9) containing 35 mg/liter kanamycin and 1 g/liter [13C]ammonium chloride as the sole nitrogen source. For U-13C,15N labeling, cells were grown at 37 °C in M9 medium containing 35 mg/liter kanamycin, 1 g/liter [15N]ammonium chloride, and 2 g/liter [13C]glucose instead of unlabeled glucose as the sole carbon source. Cells were grown to ~0.7 A600 at 37 °C, induced with 0.5 mM isopropyl-β-D-galactopyranoside, and grown overnight. Cells were harvested and resuspended in 50 mM Heps buffer, pH 7.0, containing 8 M urea and heat lysed at 100 °C for 10 min. The lysate was centrifuged, and the supernatant was loaded onto a nickel-nitrilotriacetic acid-agarose column (Qiagen). The column was washed with 50 mM Heps buffer, pH 7.0, containing 8 M urea, and the protein was allowed to re-nature in 50 mM Heps buffer, pH 7.0, on the column before eluting with 50 mM Heps, pH 7.0, containing 500 mM imidazole. Fractions containing the eluted protein were pooled and dialyzed into NMR buffer (10 mM sodium phosphate, pH 6.5, 100 mM NaCl, 0.02% (w/v) NaN3). The C-terminal His tag of the V domain was cut by thrombin (Novagen) at room temperature for 1 h before gel filtration chromatography on a S.E.-75 column (Amersham Biosciences). The fractions containing the eluted protein were concentrated to 50–500 μl by using Ultra-Centricones (Millipore). Purity was estimated to be >95% by Coomassie-stained SDS-PAGE.

Preparation of Fructosyllysine (FL) Peptides—Two model peptides containing N°-(1-deoxy-D-fructos-1-yl)-lysine (K°), AKASASFL and ALKAWSVAR, were synthesized on solid support using Fmoc chemistry following the protocol described in Frolov et al. (29).

Preparation of CML-Peptide—A model peptide, containing CML, DEF-CML-ADE peptide dissolved in D2O to be 3.92 ppm.

Preparation of CML-Bovine Serum Albumin (BSA)—We followed the protocol developed in Buettler et al. (32) that leads exclusively to CML modifications of lysine. Briefly, CML-BSA was prepared by incubating 5 mM BSA (fraction V, fatty acid-free, endotoxin free bovine serum albumin, EMD, Gibbstown, NJ) in 150 mM phosphate buffer, pH 7.4, containing 25 mM glyoxylic acid and 75 mM NaBH3CN at 50 °C for 48 h. The reaction mixture was dialyzed against 10 mM phosphate buffer, pH 7.0, and 100 mM NaCl to remove unreacted glyoxylic acid and NaBH3CN. To obtain trypsin-digested CML-BSA (trypsin-CML-BSA), 2 mM CML-BSA was incubated with 1 ml of the settled agarose gel of immobilized trypsin (Pierce) in 0.1 M NH4HCO3 buffer, pH 8.0, for 18 h at 37 °C. Immobilized trypsin was removed from the digestion mixture by centrifugation at 1000 × g for 10 min. The solution of digested AGE-BSA was dialyzed against 10 mM phosphate buffer, pH 7.0, containing 100 mM NaCl using dialysis tubing with 1-kDa molecular mass cut-off (Spectrapor, Inc). After adding 1 mM 4-(2-aminooethyl)benzenesulfonyl fluoride hydrochloride (GBT, Inc.) to inhibit the residual trypsin activity, the sample of digested AGE-BSA was used in the NMR titration experiments.

Preparation of Fruc-AGE-BSA and HOCI-BSA—Fruc-AGE-BSA was prepared as described in Valencia et al. (33). Briefly, 1 mM BSA was incubated in 400 mM phosphate buffer, pH 7.4, 0.02% NaN3, 1 mM EDTA containing 0.5 mM d-(-)-fructose as modifier for 1, 3, 6, and 12 weeks at 37 °C under sterile conditions, thus creating Fruc-AGE-BSA-1w, Fruc-AGE-BSA-3w, Fruc-AGE-BSA-6w, and Fruc-AGE-BSA-12w samples, respectively. HOCl-BSA was freshly prepared by incubating BSA with HOCI (oxidant:protein molar ratio of 100:1) in phosphate-buffered saline, pH 7.4, for 1h at 4°C (34). Low molecular weight reactants were removed by using a PD-10 column and dialysis against phosphate-buffered saline. The resultant AGE preparations were passed through a 0.22-μm filter (Millipore) to remove small particulates. Protein concentrations were determined using a Bradford assay (Pierce) according to the supplier’s protocol. The extent of modification of lysyl and arginyl residues in the AGE preparations was determined using fluorescamine and 9,10-phenanthrenequinone fluorescence-based assays (35). The lysyl residues in 1-, 3-, 6-, and 12-week Fruc-AGE-BSA preparations were estimated to be modified to 46 ± 2, 50 ± 1, 52 ± 2, and 62 ± 3%, respectively. The arginyl residues in the 1-, 3-, 6-, and 12-week Fruc-AGE-BSA preparations were estimated to be modified to 5 ± 1, 7 ± 1, 12 ± 2, and 24 ± 2%, respectively. These values are consistent with the free amine content of Fruc-AGE-BSA reported by Valencia et al. (33). The lysyl and arginyl residues in HOCl-AGE-BSA preparation were estimated to be modified to 82 ± 3 and 53 ± 1%, respectively.

**AGE-BSA Pulldown Assay**—10 μl of 10 μM His-tagged V domain of RAGE in 10 μl of binding buffer (10 mM potassium phosphate, pH 7.4, 100 mM NaCl) were mixed with 20 μl of His-tag beads (Amersham Biosciences) and equilibrated in binding buffer. 10 μl of 10 μM His-tagged C1C2 domain in binding buffer were mixed with 20 μl of His-tag beads equilibrated in binding buffer. 30 μl of 100 μM Fruc-AGE-BSA in
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binding buffer were added to the beads. The mix was incubated at 37°C for 10 min followed by 3 buffer washes. The protein absorbed on the His-tag beads was analyzed using 12% Coomassie-stained SDS-PAGE.

NMR Experiments—Protein samples of the [U-13C,15N] and [U-15N]V domain, with concentrations ranging from 60 to 100 μM, were dissolved in NMR buffer (10 mM potassium phosphate, pH 6.5, 100 mM NaCl, 0.02% (w/v) NaN3, 90%/10% H2O/D2O). The U-15N G82S V domain was dissolved in the same NMR buffer at a concentration of 30 μM. Standard triple resonance spectra 1H[15N]-HSQC, HN(CA)CO, HNCO, HN(CO)CA, HNCA, CBCA(CO)NH, and HNCACB (31) were acquired at 298 K using an Avance Bruker spectrometer operating at a 1H frequency of 700 MHz equipped with a single Z-axis gradient cryoprobe. All spectra were processed using TOPSPIN 2.1 (Bruker, Inc.) and assignments were made using CARA (36).

To study V domain-AGEs interactions, NMR titration experiments were performed. 1 mM unlabeled fructosyllysine-peptides, CML-peptide, CML-BSA, trypsin-CML-BSA, Fruc-AGE-BSA, or HOCl-BSA proteins in NMR buffer were titrated into 50 μM U-15N-labeled V domain in 6 steps to yield V domain to AGE-BSA molar ratios of 5:1, 2:1, 1:1, 1:2, 1:5, and 1:10, respectively. The results of the titration were monitored by 1H[15N]-HSQC. Over the course of titration, the signal to noise ratio of the peaks that did not show any changes was kept constant by adjusting the number of scans. The same set of NMR titration experiments with Fruc-AGE-BSA were repeated using the U-15N-labeled G82S V domain sample. Unmodified peptides, AKASASFL, ALKAWSVAR, and DEFKADE, and BSA were used as negative controls during titration experiments. No changes in the 1H[15N]-HSQC spectra of 50 μM U-15N-labeled V domain or 30 μM U-15N-labeled G82S V domain were observed up to 1 mM unmodified peptides or BSA.

Titrating AGE-BSA or HOCl-BSA into the V domain results in free V domain and a large complex, more than 100 kDa, in an exchange regime, $k_{\text{off}} = \Delta \omega_{\text{free}}$ between free and bound states. In this case we can estimate the dissociation constant, $K_d$, based on the following assumptions; for the binding reactions $k_{\text{on}}$ is usually on the order of $10^6 \text{M}^{-1}\text{s}^{-1}$. Assuming that the average change of the chemical shift is ~0.01 ppm, intermediate exchange will occur when the dissociation constant is less than or equal to 10 μM.

To measure the degree of line broadening due to complex formation we used normalized cross-peak heights (38),

$$\Delta = H_o/H_b - H_v/H_v$$

where the cross-peak heights and the average values of the cross-peak heights of the V domain are defined in the absence of AGE-BSA, $H_o$ and $H_v$ and at a given V domain to AGE-BSA ratio, $H_v$, and $H_o$, respectively. In this case large positive values of $\Delta$ indicate the resonances that are significantly broadened compared with the average value. Small positive and negative values of $\Delta$ indicate that the resonances are either broadened to the same extent or less than the average.

To monitor deuterium exchange, the reference 1H[15N]-HSQC spectrum of U-15N-labeled V domain in NMR buffer was acquired. Afterward, the NMR sample was quickly concentrated to 25 μL using a Centricone (Millipore) and diluted to 500 μL using D2O NMR buffer (10 mM potassium phosphate, pH 6.5, 100 mM NaCl, 0.02% (w/v) Na3N, 100% D2O). Ten 1H[15N]-HSQC experiments were performed to monitor deuterium exchange. Acquisition time for each experiment was about 1 h.

The influence of dynamics in the ms timescale on the relaxation properties was determined using the relaxation-compensated Carr-Purcell-Meiboom-Gill (CPMG) experiment (39). Delays between CPMG pulses ($\tau_{\text{CP}}$) of 1 and 5 ms, the relaxation delay, $T$, of 40 ms, and recycle delays of 1.5 s were used. The relaxation rates $R_{2\text{av}}$ (average between the in-phase and antiphase relaxation rates) were determined by using the relation,

$$R_{2\text{av}}(\tau_{\text{CP}}) = -1/T \times \ln(I(\tau_{\text{CP}})/I_0)$$

where $I(\tau_{\text{CP}})$ is the intensity of a peak at delay $\tau_{\text{CP}}$, and $I_0$ is the intensity of the same peak in the reference spectrum. No attempt was made to extract the exact rates from the limited data. Instead, residues that showed a statistically significant difference, $\Delta R_{2\text{av}} > 6.5 \text{s}^{-1}$ between the relaxation rates measured using $\tau_{\text{CP}} = 1$ ms and $\tau_{\text{CP}} = 5$ ms, were identified as displaying dynamics on the ms timescale.

To measure residual dipolar couplings, 0.1 mM U-15N-labeled V domain in NMR buffer was diazylated into an 8% polycrylamide stretched gel (acylamide/bisacylamide ratio 49:1) (40). The observed quadrupolar splitting of the solvent 2H signal in the stretched gel was 9 Hz. The N–H residual dipolar couplings ($D_{\text{NH}}$) were measured using the 1H[15N]-HSQC-IPAP sequence. $D_{\text{NH}}$ values were calculated as the difference between splittings measured in an aligned sample and an isotropic sample. Errors in the measurement of dipolar couplings were estimated from the ratio of line-width to signal-to-noise (with the addition of errors from each contributing peak) at 0.5 Hz for N–HN couplings.
Homology Modeling—Homology modeling of the V domain structure was performed using the Swiss-Model optimization mode prepared by Swiss-PDB Viewer (41). Sequence homology between the V domain and the immunoglobulin domain from murine JAM1 (PDB code 1F97) (42) was based on alignment of conserved residues and the homology of secondary structures as revealed by NMR experiments. Energy minimization was performed using GROMOS96 with successive application of 200 cycles of steepest descent and 300 cycles of conjugate gradient minimizers. The quality of the resultant structure was estimated by the PROCHECK program (43). 92% of the V domain residues fall into the most favorable region of the Ramachandran plot, and 8% fall to the allowed region. A backbone atom root mean square deviation of 1.5 Å was calculated by comparing the model to the JAM1 crystal structure.

Dipolar couplings were fit to the structural model of the V domain using singular value decomposition (44) in the program PALES (45). The Pearson product-moment correlation coefficient between experimentally determined $D_{NH}$ and $D_{NN}$ calculated based on the structural model of the V domain was obtained using PALES.

RESULTS

Unligated RAGE Forms Homooligomers on the Plasma Membrane of Living Cells—We used confocal microscopy to verify that the expressed constructs of RAGE are properly targeted to the plasma membrane. To visualize RAGE in living cells, we tagged RAGE at the C terminus with CFP (RAGE-CFP) or yellow fluorescent protein (RAGE-YFP) and expressed these proteins in HEK 293T cells. Previously, HEK293T cells expressing RAGE were used to study RAGE signaling (2). RAGE is clearly localized on the plasma membrane consistent with previous studies (2, 46, 47) (Fig. 1).

Previous results indicated that bacterially (14) and eukaryotically (48) overexpressed sRAGE is a constitutive oligomer. We performed FRET experiments to determine whether RAGE forms homooligomers on the plasma membrane of living cells (Fig. 1 and supplemental Fig. 1). FRET is a nonradiative phenomenon in which energy is transferred from a donor fluorophore to an acceptor chromophore with an efficiency that depends on the distance between the two chromophores, the extent of overlap between the donor emission and acceptor excitation spectra, the quantum yield of the donor, and the relative orientation of the donor and acceptor (49). If two RAGE molecules labeled with CFP and YFP are located within 100 Å from each other, the energy emitted by the donor (RAGE-CFP) (in its excited state) will be transferred to the acceptor (RAGE-YFP), resulting in acceptor excitation and quenching of donor fluorescence. When the acceptor is removed by photobleaching, the donor is dequenched, and an increase in donor fluorescence is observed (27) (Fig. 1). Laser-scanning confocal microscopy allowed the photobleaching to be confined to a small region of plasma membrane, minimizing the time required for photobleaching and making the technique suitable for living cells.

We used monomeric versions of the fluorescence proteins, mCFP and mYFP (26), to avoid nonspecific oligomerization of RAGE caused by the inherent ability of CFP and YFP to dimerize at high concentrations (26). In a typical experiment, a selected region of plasma membrane of a living HEK293T cell co-expressing RAGE-mCFP and RAGE-mYFP (indicated by white boxes in Fig. 1) was photobleached. Pre-bleach (Fig. 1, A–C) and post-bleach (Fig. 1, D–F) images were captured (Fig. 1). FRET efficiency was calculated as an increase in CFP (donor) fluorescence (Fig. 1, A and D, and supplemental Fig. 1) due to YFP (acceptor) photobleaching (Fig. 1, B and E). Data collected from more than 70 cells demonstrate the average FRET efficiency for RAGE to be 28 ± 4%, whereas acceptor photobleaching typically results in over a 90% decrease in fluorescence. To estimate the error margin contributed to FRET by lateral protein movements within the membrane, FRET efficiency was determined for HEK293 cells expressing only RAGE-mCFP and was found to range from −3.1% to +1.5%. Similar values were obtained for the non-bleached membrane regions, indicating that receptor movements into and out of the region of interest during imaging do not significantly distort our FRET efficiency data.

It is important to distinguish between a FRET signal produced by specific protein-protein interactions, resulting in dimers or higher order oligomers, and a false positive FRET...
signal resulting from random proximity of the donor and acceptor due to overexpression (50, 51). FRET produced by oligomeric proteins is dependent on the donor:acceptor ratio, which is calculated using unquenched post-bleached donor fluorescence divided by pre-bleached acceptor fluorescence, uD/A, and independent of the acceptor expression level. Conversely, a FRET signal resulting from random proximity is dependent on the amount of acceptor expressed on the plasma membrane. To rule out a false positive FRET effect, we plotted FRET efficiencies, measured in cells co-expressing RAGE-mCFP and RAGE-mYFP, versus uD/A ratios and the amount of acceptor overexpressed on the plasma membrane. The FRET efficiency is clearly dependent on the uD/A ratios (Fig. 2A) and independent of the level of acceptor expression (Fig. 2B) as is the case for oligomeric proteins. This result proves that the FRET signal is due to specific protein-protein interactions and is not due to overexpressing RAGE. Together these results provide strong evidence that RAGE forms homooligomers on the plasma membrane of HEK293T cells.

**NMR-based Structural Model of the V Domain of RAGE**—To demonstrate that overexpressed V domain binds biologically relevant ligands, we used a pulldown assay. We established that only the V-domain of RAGE binds to fructose AGE-BSA (Fruc-AGE-BSA) (supplemental Fig. 2), glyoxal AGE-BSA (GA-AGE-BSA) (data not shown), and hypochlorite-modified BSA, HOCl-BSA (data not shown). A construct consisting of the tandem C1C2 domains of RAGE did not bind to any of the various AGE-BSAs. These results are consistent with experiments that show the V domain of RAGE can successfully compete with sRAGE for AGES binding (2).

Atomic resolution information about the interaction surface between RAGE and AGES can be obtained using heteronuclear NMR spectroscopy (52, 53). To facilitate NMR binding studies between the V-domain of RAGE and AGES, we first had to assign the chemical shifts of the V-domain of RAGE. Backbone assignments of the human V-domain of RAGE were made using uniformly labeled U-13C,15N protein, overexpressed in *E. coli* using a T7 expression system. The purified protein has a primary sequence of 102 amino acids with the first methionine removed by bacterial aminopeptidase. The V domain is predominantly monomeric in solution (14, 54). Sequential assignments of the V-domain of RAGE were achieved using HNCA/HN(CO)CA, HNCO/HN(CA)CO, and HN(CA)CB/HN(CO)CB pairs of triple resonance NMR experiments (31) so that connectivities could be traced through three independent, through-bond pathways. Overall, 91% of the 1H, 13C, 13CO, and 15N backbone resonances were sequentially assigned (Fig. 3A). The incompleteness of the assignments can be traced to broadening of the peaks due to amide proton exchange processes in the loop structures and structural heterogeneity of the β strands exhibited in the irregular shapes of the NMR peaks. Despite these gaps, the majority of backbone resonance assignments are complete for the V domain of RAGE, providing site-specific reporters for ligand binding.

To show that the immunoglobulin-like V domain has a properly formed disulfide bond between Cys-38 and Cys-99, we assigned the 13C of Cys-38 and Cys-99 to be 41 and 39 ppm, respectively. The 13C chemical shift is a very sensitive probe of the redox state of Cys as there is a large spectral separation (10 ppm) between the reduced and oxidized states of Cys (31). We did not see any evidence of reduced Cys-38 or Cys-99 that would have a 13C chemical shift resonance at around 30 ppm.
We conclude that the V domain does not have any heterogeneity due to the disulfide bond.

The secondary structure of the V domain (Fig. 3B) is based on the PECAN algorithm (55), which utilizes the amino acid sequence and NMR chemical shifts to determine elements of protein secondary structure (supplemental Fig. 3). Assignments of the amides that participate in hydrogen bonding to form β-sheets were confirmed by measuring the rate of hydrogen-deuterium exchange of the backbone amides. After 1 h at room temperature, only hydrogen-bonded amides of the V domain remained in the 1H{15N}-HSQC spectrum. After 2 h, all amide peaks disappeared from the 1H{15N}-HSQC spectrum, suggesting a very high H-D exchange rate and implying that deuterium can reach partially and completely buried amide hydrogens and even hydrogen-bonded amides. This observation is functionally important as it suggests that the V domain has a high degree of flexibility.

Previously, flexible regions were thought to be involved in ligand binding. To further characterize the flexibility of the V-domain, we performed CPMG NMR relaxation experiments (39). These experiments are designed to identify amide backbone protons that undergo dynamic fluctuation on the ms to μs timescale. Residues Thr-27, Arg-48, Leu-53, Arg-57, Glu-59, Gln-67, Arg-73, Leu-86, Phe-97, Arg-98, and Gln-119 exhibit motions on this timescale. Thr-27, Leu-53, Glu-59, Phe-97 and Arg-98 are located in the middle of the β strands A′, C′, C′, F, and F, respectively, suggesting that these strands undergo large structural changes (Fig. 4). Arg-48, Arg-57, Gln-67, Asp73, Leu-86, and Gln-119 are located either within or at the ends of flexible loops.

The extremely dynamic nature of the V domain precludes collecting the high quality nuclear Overhauser effect spectroscopy spectra necessary to solve the high resolution structure of the molecule by using NMR spectroscopy. Instead, we decided to construct a structural model of the V domain that correctly reflects the NMR observables (Fig. 4). The V domain belongs to the variable type immunoglobulin domain family (56). There is an extensive data base of immunoglobulin-like structures available to generate a high quality model. We used the primary and secondary structures of the V domain of RAGE to search for the appropriate structural template for homology modeling.

A structural model of the V domain (Fig. 4) was generated using the structure of the junction adhesion molecule, JAM1 (PDB code 1F97) (42). Despite low (30%) sequence identity between murine JAM1 and the V domain of RAGE, secondary elements of both proteins match (Fig. 3B). To further validate the structural model we determined residual dipolar couplings (RDC) between backbone amide protons and nitrogens using a weakly aligned NMR sample of the V domain (57). RDCs depend on the angle between a NH bond and the protein alignment axis and can serve as quality factors for high resolution structures as well as structural models (58). Overall, 54 NH

![FIGURE 3. NMR spectrum and primary sequence alignment of the V domain of RAGE. A, 1H{15N}-HSQC spectrum of the U-15N-labeled V domain of RAGE showing resonance assignments of the backbone amide protons and nitrogens. B, primary sequence alignment of the V domain of RAGE and an immunoglobulin domain of murine JAM1 (sequence accession code O88792). Sequence numbers are indicated on the sides. The identical residues are highlighted in red, and similar residues are shown in blue. Secondary structure elements of the V domain are shown above the sequence. Secondary structure elements are based on NMR chemical shifts indices (78) and hydrogen-deuterium exchange experiments.](image-url)
dipolar couplings of the V domain were determined. The correspondences of the measured dipolar couplings to those calculated based on the V domain structural model were assessed using Pearson product-moment correlation coefficients. The value of Pearson product-moment correlation coefficient, 0.81, suggests that our structural model correctly reflects the overall backbone orientation of the V domain (supplemental Fig. 4).

The G82S Mutation Destabilizes the Tertiary Structure of the V Domain—Close examination of the $^1H{^{15}N}$-HSQC spectrum of the V domain revealed that there is an additional set of minor peaks, which are 10% intensity that of the major V domain peaks (Fig. 3). These peaks have poor amide proton dispersion from 8.5 to 7.5 ppm, which indicates unstructured protein. We assumed that the expressed V domain exists in solution in two differentially populated states, a folded state and an unfolded state. The major peaks seen in the $^1H{^{15}N}$-HSQC spectrum are from the folded state, and the minor peaks are from the unfolded state. A similar situation was also described for the unrelated DrkN SH3 domain (59). To prove that folded and unfolded states are in thermodynamic equilibrium, we obtained an NMR spectrum in the presence of sodium sulfate, which is known to stabilize folded protein structures. Indeed, at 200 mM sodium sulfate, the relative intensity of the peaks from the unfolded state of the V domain decreased from 10% to around 5% (data not shown).

Compared with wild type, the $^1H{^{15}N}$-HSQC spectrum of the G82S mutant exhibited a reverse in population of the major and minor peaks (Fig. 5, Detail A), suggesting that the unfolded state dominates the NMR spectrum. The major peaks of the G82S mutant show poor amide proton dispersion, and the minor peaks, which are about 10% intensity of the major...
peaks, are well dispersed. We assumed that the G82S mutation caused a major destabilization of the folded state of the V domain. The chemical shifts of the minor peaks of the G82S mutant are very close to the major peaks of the wild type V domain, suggesting that the overall structure of the folded state of the G82S mutant did not undergo a significant change. Adding 200 mM sodium sulfate increased the population of the folded form of G82S mutant (Fig. 5, Detail B). Therefore, the folded and unfolded states of the G82S mutant are in thermodynamic equilibrium, with the unfolded state dominating the population.

Interaction between the V Domain of RAGE and Chemically Defined AGEs—The structural heterogeneity of AGEs is a major obstacle to performing high resolution studies of the AGEs-RAGE interaction. We approached this problem by first characterizing the interaction between RAGE and a small subset of chemically defined structures found in glycated proteins.

FL is an early Amadori product formed during the reaction between proteins and D-glucose (60). FL is the predominant early glycation modification found in vivo. We synthetically incorporated this lysine modification into the model peptides, AK*ASASFL and ALK*AWSVAR, where K* denotes fructosyllysine. We then used NMR spectroscopy to test the ability of these peptides to bind to the V domain.

Unlabeled FL peptides were titrated into a U-15N-labeled V domain solution, and protein-ligand interactions were monitored using 1H{15N}-HSQC experiments. Chemical shifts of the protein backbone nuclei observed by using NMR spectroscopy are exquisitely sensitive to any structural changes that occur during ligand binding (53). Monitoring chemical shift changes during the titration allows us to define the protein-ligand interaction surface with atomic resolution. We observed no changes in the 1H{15N}-HSQC spectrum of the 50 mM U-15N-labeled V domain solution after adding up to 1 mM concentration of either FL peptide. We concluded that early glycation products do not contribute to the ability of glycated proteins to bind to RAGE.

CML and CEL are also major amino acid modifications found in AGE proteins (61). We examined the ability of CML and CEL to bind to the V domain using NMR spectroscopy. We observed no changes in the 1H(15N)-HSQC spectrum of the 50 μM U-15N-labeled V domain solution after adding up to 10 mM concentrations of either CML or CEL. This observation suggests that free modified amino acids do not bind to the V domain, consistent with the results from the competition experiment between free CML and the sRAGE-AGE-BSA complex (7). We concluded that incorporating CML into a peptidic structure may be required for binding to the V domain.

We followed the protocol developed in Gruber and Hofmann (30) to incorporate CML into a specific position of a model CML-peptide from BSA, DEF-CML-ADE. Titrating the CML-peptide into the 50 μM U-15N-labeled V domain solution resulted in gradual chemical shift changes of the 1H(15N)-HSQC spectrum, suggesting a fast chemical exchange regime between the CML-peptide and the V domain. Based on the titration data, we estimated that the V domain affinity for the CML-peptide is 103 ± 22 μM. Seven distinct regions of secondary structure are affected by the CML-peptide binding: strand A’ (Arg-29 and Ile-91), strand C (Cys-38), loop CC’ (Gly-40), strand C’ (Leu-49), loop FG (Arg-98), and loop FG (Asn-105 and Gly-106) (supplemental Fig. 5A and Fig. 6A). Most amino acid residues affected by the CML binding are located on the surface of the V domain, suggesting that the observed chemical shift changes are due to the direct interaction between the CML-peptide and V domain. Mapping these residues onto the molecular surface of the V domain allowed us to identify three interaction surfaces involved in binding the CML-peptide. The first interaction surface (IS1) consists of strand C and loop CC’, the second interaction surface consists of strand F and loop FG, and the third interaction surface consists of strand A’ and loop EF (Fig. 6A). These surfaces are
located away from each other and presumably bind CML-peptide independently from each other.

To investigate the importance of the peptide primary structure on V domain recognition, we used the protocol developed in Buetler et al. (32) to make CML-modified BSA (CML-BSA). This protocol results in predominantly CML modification of lysines. Trypsin digestion of CML-BSA created multiple peptides with CML incorporated into various primary structures (trypsin-CML-BSA). Titrating trypsin-CML-BSA into the 50 μM U-15N-labeled V domain solution also resulted in gradual chemical shift changes of the 1H-15N-HSQC spectrum (supplemental Fig. 5B). The trypsin-CML-BSA-V domain interaction surface was similar to the CML-peptide-V domain interaction surface (Fig. 6B).

There were few additional amino acids that were significantly shifted in the latter case: Leu-36 in strand C, Glu-50 in strand C', Glu-94 in loop EF, and Met-102 in strand F. All these amino acids are located in the immediate vicinity of the IS1, IS2, or IS3 surfaces (Fig. 6B).

We also investigated whether protein tertiary structure leads to a change in the interaction surface between CML-containing proteins and the V domain by titrating unlabeled CML-BSA into the 50 μM U-15N-labeled V domain solution. As in the case of CML-peptide and trypsin-CML-BSA, we observed gradual chemical shift changes upon increasing the CML-BSA concentration. We estimated the V domain binding affinity for CML-BSA to be 120 ± 20 μM, which is very similar that of the CML-peptide. Six distinct regions of secondary structure are affected by CML-BSA binding: strand C (Leu-36 and Cys-38), loop CC' (Gly-40 and Ala-41), strand C' (Arg-48, Leu-49, and Glu-50), loop EF (Ile-91), strand F (Gly-95, Arg-98, and Cys-99), and loop FG (Asn-105, Gly-106, and Lys-107) (supplemental Fig. 6A). Compared with CML-peptide binding, strand A' is not on the interaction interface. At the same time, strands C' and F had additional residues, Arg-48, Glu-50, Arg-98 and Cys-99, which changed their chemical shifts upon binding CML-BSA. The interaction surface of the CML-BSA-V domain complex consisted largely of extended IS1 and IS2 regions (Fig. 7A). The IS3 surface was significantly smaller than during interactions with the CML-peptide or trypsin-CML-BSA.

Interaction between the V Domain of RAGE and Fruc-AGE-BSA and HOCl-BSA—Having established that CML-modified BSA is a low affinity ligand for the V domain, we asked whether there are other AGE structures that contribute to binding to RAGE.

D-Glucose plays a primary role in glycation of proteins in vivo due to its high concentration in human plasma. At the same time, it is among the least reactive reducing sugars in biological systems. To avoid the long incubation times needed to generate AGE-proteins in vitro we used D(-)-fructose during the glycation reaction. D(-)-Fructose is about 8-fold more reactive than glucose and is known to be elevated to glucose levels in a number of tissues of diabetic patients (62). D(-)-Fructose-modified BSA (Fruc-AGE-BSA) is well characterized and widely used for RAGE binding studies (7, 63). D(-)-Fructose-modified BSA (Fruc-AGE-BSA) results in preferential formation of CML and extensive cross-linking of the protein (61) (supplemental Fig. 2, left). Depending on the BSA reaction time with D(-)-fructose, Fruc-AGE-BSA will possess different sets of either early glycation or AGE structures that may change its ability to bind RAGE. Following standard protocols (7, 34, 64), we made four...
different preparations of Fruc-AGE-BSA by incubating BSA in 0.5 M D-(−)-fructose solution for 1, 3, 6, and 12 weeks, creating Fruc-AGE-BSA-1w, Fruc-AGE-BSA-3w, Fruc-AGE-BSA-6w, and Fruc-AGE-BSA-6w samples, respectively.

Hypochlorous acid-modified BSA was also characterized to be a high affinity ligand of RAGE (34). Short, 30-min, exposure of BSA to hypochlorous acid does not result in the formation of AGEs but, rather, forms stable epsilon chloramines with lysine side chains and leads to oxidation of cysteine and methionine side chains (64). There is significant cross-linking formed during hypochlorous acid modification of BSA (supplemental Fig. 2, left).

NMR spectroscopy can resolve the problem resulting from structural heterogeneity of AGEs produced by reducing sugars or exposure to hypochlorous acid as long as the U-15N-labeled protein is homogeneous. The selectivity of isotope edited NMR experiments is such that one can study protein-ligand interactions involving 15N-labeled proteins even inside a crowded cellular cytosol (65, 66). By employing 15N-edited NMR experiments we are able to see changes in the interaction surface of the V domain of RAGE elicited by a diverse set of AGE-BSA and HOCl-BSA ligands.

The V domain-Fruc-AGE-BSA complexes are highly heterogeneous, have a large (>100 kDa) molecular mass, and probably are invisible by NMR. Nevertheless, they can be observed indirectly by observing the changes in the position and line-width of the free V domain chemical shifts due to the exchange with the V domain-Fruc-AGE-BSA complex (see “Experimental Procedures”). Chemical exchange detected by NMR spectroscopy is generally characterized by three regimes: fast exchange when the characteristic exchange rate, \( k_{ex} \), is much larger than the changes in chemical shifts between free and bound forms, \( \Delta \omega_{ex} \), intermediate exchange when \( \Delta \omega_{ex} \approx \Delta \omega_{ex} \), and slow exchange when \( \Delta \omega_{ex} \approx \Delta \omega_{ex} \). In the fast exchange regime, even in the case of large protein-protein complexes, we should see gradual changes in the chemical shifts of the V domain nuclei affected by protein-protein binding. In the slow and intermediate exchange regime, we will see differential broadening of the V domain NMR signals originating from the affected nuclei (52, 69, 70).

No changes in the NMR spectrum of U-15N-labeled V domain were observed by titrating up to 1 mM Fruc-AGE-BSA-1w into a 50 \( \mu \)M U-15N-labeled V domain solution. We assumed that the majority of lysines in Fruc-AGE-BSA-1w were modified with early glycation products that do not bind to RAGE.

Titrating Fruc-AGE-BSA-3w into a 50 \( \mu \)M U-15N-labeled V domain solution resulted in differential broadening and gradual chemical shift changes of a number of backbone amide peaks. The binding affinity of the V domain for Fruc-AGE-BSA was estimated to be about 10 \( \mu \)M (“Experimental Procedures”). Among the amino acids exhibiting extreme differential broadening are Arg-29, Ile-30, Glu-50, Ile-91, and Glu-94 (supplemental Fig. 6B). As the concentration of Fruc-AGE-BSA increases, Leu-36, Lys-37, Cys-38, Gly-40, Ala-41, Lys-43, Leu-49, Arg-48, Arg-98, Asn-105, and Gly-106 undergo gradual change in chemical shifts. Seven distinct regions of secondary structure are affected by Fruc-AGE-BSA-3w binding: strand A’ (Arg-29 and Ile-30), strand C (Leu-36, Lys-37, and Cys-38), loop CC’ (Gly-40, Ala-41 and Lys-43), strand C’ (Arg-48, Leu-49, and Glu-50), loop EF (Ile-91 and Glu-94), strand F (Arg-98), and loop FG (Asn-105, Gly-106, and Lys-107) (supplemental Fig. 6B and Fig. 7B). Mapping these residues onto the molecular surface of the V domain revealed that the Fruc-AGE-BSA-3w-V domain interaction surface is similar to that identified for CML-peptide-V domain interactions (Fig. 7, A and B). The same three areas of the V domain are involved in binding Fruc-AGE-BSA-3w (Fig. 7B). The IS1 consists of strand C and loop CC’, the second interaction surface consists of strand C’, strand F, and loop EF, and the third interaction surface consists of strand A’ and loop EF. IS1 and IS3 surface areas of Fruc-AGE-BSA-V domain interaction are significantly larger than those in the case of CML-BSA, presumably due to the involvement of cross-linking structures of Fruc-AGE-BSA (supplemental Fig. 2, left) in the V domain binding.

Titrating Fruc-AGE-BSA-6w and Fruc-AGE-BSA-12w into a 50 \( \mu \)M U-15N-labeled V domain solution resulted in differential broadening and chemical shift changes of the backbone amide peaks that are identical to that obtained during Fruc-AGE-BSA-3w titration (data not shown). We assumed that no new AGE structures were generated over the course of prolonged incubation with D-(−)-fructose that exhibit significant binding to the V domain.

Titrating HOCl-BSA into a 50 \( \mu \)M U-15N-labeled V domain solution resulted in chemical shift changes of a number of backbone amide peaks (supplemental Fig. 6C). We estimated that the V domain affinity for HOCl-BSA is 93 ± 15 \( \mu \)M (supplemental Fig. 7). The interaction surface of the V domain affected by HOCl-BSA binding is located in IS1 (Fig. 7C). The loop CC’ (Ala-41) and strand C’ (Leu-49) are predominantly involved in this interaction (supplemental Fig. 6C).

Titrating Fruc-AGE-BSA-3w or CML-BSA into a 30 \( \mu \)M U-15N-labeled G82S V domain solution revealed a complex picture for the AGES G82S V domain interaction. The AGES G82S V domain complex is in slow exchange with the free components, indicating that the binding affinity is tighter than 1 \( \mu \)M. This slow exchange rate between AGES-BSA and the V domain prevents surface mapping using a chemical shift perturbation technique. As the concentration of AGES-BSA increased, the peaks from the folded state of the G82S V domain uniformly disappeared. Peaks from the unfolded state of G82S RAGE exhibited chemical shift changes as well as a slow uniform decrease in intensities. We assumed that it is the folded state of the G82S V domain that predominantly interacts with AGES-BSA.

**DISCUSSION**

In the present study we structurally characterized interactions between the multiligand receptor, RAGE, and a diverse set of its ligands created by non-enzymatic glycation of extracellular proteins. RAGE presents a particular challenge to the well established concept of molecular recognition stating that every receptor has a ligand that binds to a specific area on its surface to invoke physiological responses (71). Every intercellular protein can be AGE-modified with a diverse set of chemical adducts creating potential ligands for RAGE. The question is...
how this myriad of seemingly unrelated chemical structures can be recognized by one receptor?

To answer this question we first confirmed that the V domain of RAGE is solely responsible for binding AGE-modified proteins. Our attempts to obtain a high resolution solution structure of the V domain were hampered by broadening of a large number of NMR peaks. We established that the spectral broadening is predominantly due to large ms-μs fluctuations in strands A, C, C", and F and the loop structures of the V domain, which restructure a significant portion of the V domain surface. This structural plasticity may be important for adapting the binding surface to different classes of RAGE ligands.

Our observation of the limited structural stability of the V domain was in agreement with the results reported in Dattilo et al. (15), in which the thermodynamic stability of the V domain and VC1 tandem construct of RAGE was thoroughly studied using differential scanning calorimetry and trypsin digestion. It was shown that the V domain acquires additional stability in the context of the VC1 construct due to the specific structural interface between the V and C1 domains. At the same time, comparing the NMR spectra of the V and VC1 domains revealed that only a small number of peaks corresponding to the V domain backbone protons and nitrogens change their positions. The observed chemical shift changes were less than 0.1 ppm, indicating that the structure of the V domain is largely preserved in the context of the larger constructs.

We constructed a structural model of the V domain based on chemical shift assignments and the location of hydrogen bonds determined by using NMR spectroscopy. RDCs of the weakly oriented protein provided a useful tool for validating the quality of the structural models versus NMR data (58). The measured RDCs of the NH bonds of the V domain were compared with RDCs calculated based on the structural model of the V domain to show that the model is a good approximation of the solution structure of the V domain.

To study structural mechanisms of AGEs interacting with the V domain we used two different types of AGE ligands, the FL- and CML-peptides and CML-BSA, containing two major chemically defined structures of early and advanced glycation end products found in proteins FL and CML, respectively, and a heterogeneous set of AGE ligands created by incubating BSA in d-fructose solution for varying times, Fruc-AGE-BSA-1w, Fruc-AGE-BSA-3w, Fruc-AGE-BSA-6w, and Fruc-AGE-BSA-12w. Fruc-AGE-BSA-1w possesses mostly early glycation products (61). Fruc-AGE-BSA-3w, -6w, and -12w ligands possess predominantly CML modifications (61) and over longer fructosylation times are significantly cross-linked (supplemental Fig. 2, left). We also used hypochlorous acid-modified BSA, HOCl-BSA, which does not contain AGEs but was shown to contain a large amount of stable ε-chloramines and strongly binds to RAGE (34). Ligands containing the early glycation products FL-peptide and Fruc-AGE-BSA-1w did not bind to the V domain up to millimolar concentrations. We concluded that early glycation products are not recognized by RAGE. On the other hand, the remaining preparations interacted with the V domain with affinities ranging from 10 μM for Fruc-AGE-BSA to 100 μM for CML-peptide, CML-BSA, and also HOCl-BSA.

We characterized the interaction surfaces of the V domain that bind various preparations of AGE-BSAs by using an ultra-sensitive NMR chemical shift perturbations technique (53). The V domain residues affected by interaction with AGE-BSA lie on the protein surface, suggesting that there is no major conformational change upon complex formation. Using chemically defined CML-peptide as a V domain ligand, we established that three distinct surfaces, located in highly positively charged areas of the V domain, are involved in the CML-peptide binding. We also established that the primary sequence of the CML-peptide as well as the location of CML within the folded protein does not play a major role in recognition since both trypsin-digested CML-BSA and CML-BSA bind to very similar regions of the V domain. At the same time, our failure to detect any changes in the NMR spectra when adding free CML and CEL suggests that in addition to side chain modifications, AGE-modified amino acids have to be embedded in strongly negatively charged areas of the AGE-protein to be able to effectively interact with RAGE.

The interaction surface between Fruc-AGE-BSA-3w and the V domain encompasses all three regions of the V domain, IS1, IS2, and IS3, involved in binding CML-containing ligands. This was expected since the major modification found in Fruc-AGE-BSA-3w was CML (61). At the same time, compared with CML-BSA, two interaction surfaces, IS1 and IS3, spanned larger surface areas (Fig. 7, A and B), suggesting the importance of other AGE structures, possibly cross-links, in V domain binding. The observation of larger interaction surfaces tracks with the increase in binding affinity of the V domain for Fruc-AGE-BSA-3w versus CML-BSA from about 100 to 10 μM. The interaction surfaces of Fruc-AGE-BSA-6w and Fruc-AGE-BSA-12w with the V domain were identical to those of the Fruc-AGE-BSA-3w-V domain interaction surfaces. This result suggests that AGE structures responsible for the V domain binding were already present after 3 weeks of BSA incubation with d-fructose. It also argues for the fact that the V domain recognizes individual AGE structures and that V domain binding does not depend on the extent of AGE modifications of the proteins. This is especially important because physiologically relevant AGEs are found to be modified to a significantly smaller degree (1 mmol of AGE per mol of lysine) than those used for the NMR experiments in the present study (0.5 mol of AGE per mol of lysine) (72).

The HOCl-BSA-V domain complex exhibits a small interaction surface that is located within IS1. Chloramine lysine is the predominant modification present in HOCl-BSA (73). Modifications of methionine, tyrosine, and cysteine residues may also be present. It is interesting that although these modifications are distinctly different from AGEs, they are recognized by one of the V domain-AGEs interaction surfaces. This fact underscores the structural plasticity of the V domain in recognizing RAGE ligands.

Recently, two peptide sequences from the V domain were shown to interact with specific preparations of AGE-BSA (19, 74). The first peptide, (19) PEP1 (amino acids 39–54), includes residues in IS1 and IS2, identified here by chemical shift map-
ping. Using a surface plasmon resonance binding assay, PEP1 was shown to have micromolar affinity for glyceraldehyde-derived AGE-BSA and nanomolar affinity for glycolaldehyde-derived AGE-BSA. The second peptide, PEP2 (amino acids 102–118), includes residues in IS2 (74). In a functional cell-based assay, PEP2 neutralized CML-human serum albumin activity induced in THP-1 cells in a dose-dependent manner.

Our characterization of the G82S V domain showed that G82S polymorphism leads not only to a local change around the mutation site but also to a global destabilization of the tertiary structure of the protein. According to the NMR analysis, there are two major conformations of the G82S V domain, folded and unfolded, which exist in thermodynamic equilibrium. The unfolded conformation of the G82S V domain is at least 10 times more populated than the folded conformation. At the same time, the G82S mutation leads to an increase in the binding affinity of the V domain for AGE-BSA (18, 74), possibly, due to the enhanced flexibility related to the existence of two conformers.

The binding affinity of the monomeric V domain for AGE-BSA, $K_{d,V} \sim 10 \mu\text{m}$, is much weaker than that reported for sRAGE or full-length RAGE, $K_{d,RAGE} \sim 90 \text{nm}$ (7). We think that the discrepancy in binding affinity is due to constitutive oligomerization of RAGE that brings together several V domains, thereby increasing the number of sites available for binding. AGE-BSA usually has multiple AGE-modified sites within one molecule, and experimental results from surface plasmon resonance measurements of AGE-BSA binding to immobilized sRAGE showed that only cross-linked, high molecular weight AGE complexes were able to interact with sRAGE (75). In addition, proteolytic digestion of AGE complexes caused the loss of RAGE binding capability (75). These observations imply that multiple AGE-modified side chains are required for high affinity interactions, and oligomerization of RAGE provides a mechanism to increase the number of V domain binding sites available to bind AGE modified side chains.

Using fluorescence energy transfer, we showed that RAGE is indeed a constitutive oligomer when expressed on the surface of HEK293 cells. Previously, we showed that bacterially expressed sRAGE is a tight tetramer and the C1 domain is responsible for this oligomerization (14). The increased binding affinity of sRAGE for S100B tetramers versus dimmers (54) also supports our conclusion about the importance of constitutive oligomers of sRAGE for ligand binding.

Using thermodynamic considerations, we can rationalize the importance of RAGE oligomerization in its function as a pattern recognition receptor (Fig. 8) assuming that, on average, only two V domains are engaged by AGE-BSA at a time. Following the formalism of Jencks (76), we can present the free energy of binding AGE-BSA to oligomerized RAGE, $\Delta G_{RAGE}$, as the sum of the intrinsic free energies of AGE-BSA binding to two isolated V domains, $\Delta G_{i}$, plus a connection free energy, $\Delta G_{c}$, that represents the change in the probability of binding due to the oligomerization,

$$\Delta G_{RAGE} = 2\Delta G_{i} + \Delta G_{c}$$  \hspace{1cm} (Eq. 5)\

$\Delta G_{c}$ has a large entropic component that corresponds to changes in translational and rotational degrees of freedom and includes any free energy required to bring about conformational change in oligomeric RAGE that accompany AGE-BSA binding.

The magnitude of $\Delta G_{c}$ can be estimated by substituting $\Delta G_{i} = -RT\ln(K_{i}^{\text{RAGE}})$ and $\Delta G_{RAGE} = -RT\ln(K_{d}^{\text{RAGE}})$ into Equation 1, where $K_{d}^{\text{RAGE}} = 1/K_{d}^{\text{RAGE}}$ and $K_{d}^{\text{V}} = 1/K_{d}^{\text{V}}$.

$$\Delta G_{c} = -RT\ln((K_{c}^{\text{V}})/(K_{c}^{\text{RAGE}}))$$  \hspace{1cm} (Eq. 6)

Given dissociation constants of 10 $\mu\text{m}$ (this work) and 90 nm (7) for binding AGE-BSA to the isolated V domain and to oligomeric RAGE, respectively, the connection free energy equals $+4.0 \text{ kcal/mol}$. The positive value of this compensatory term indicates tertiary or quaternary strain and a loss of binding entropy, possibly due to steric hindrance and/or suboptimal arrangement of the AGE-BSA binding sites.

However, the overall binding free energy of oligomeric RAGE for AGE-BSA ($\Delta G_{RAGE} = -9.4 \text{ kcal/mol}$) is significantly stronger than the binding free energy of the individual V domain for AGE-BSA ($\Delta G_{i} = -6.7 \text{ kcal/mole}$), reflecting the energetic advantage of bringing two V domains together and the ability of AGE-BSA to bind to more than one V domain simultaneously. Thus, the low affinity/high specificity binding of AGE-BSA to an individual V domain is transformed into high affinity/high specificity binding as a result of RAGE oligomerization.

We propose that augmented binding energy due to the oligomerization of low affinity monomeric subunits and the structural heterogeneity of AGE ligands, which present multiple determinants capable of binding to different interaction surfaces on the V domain, provide a general mechanism for molecular pattern recognition by RAGE (Fig. 7). The combination of these factors may result in a signal competent conformation of RAGE or may inhibit productive sig-
naling. Similarly, the interplay between binding at different interaction surfaces and different chemical structures of AGE ligands may be responsible for the reported failures to detect RAGE signaling by some specific AGEs (32, 77). This mechanism presents new opportunities for controlling RAGE signaling by screening for small molecules that interfere with the AGE interaction surfaces of the V domain delineated in this study.

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