The G82S Polymorphism Promotes Glycosylation of the Receptor for Advanced Glycation End Products (RAGE) at Asparagine 81

Comparision of Wild-Type RAGE with the G82S Polymorphic Variant

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Interaction between the receptor for advanced glycation end products (RAGE) and its ligands amplifies the proinflammatory response. N-Linked glycosylation of RAGE plays an important role in the regulation of ligand binding. Two potential sites for N-linked glycosylation, at Asn25 and Asn81, are implicated, one of which is potentially influenced by a naturally occurring polymorphism that substitutes Gly82 with Ser. This G82S polymorphic RAGE variant displays increased ligand binding and downstream signaling. We hypothesized that the G82S polymorphism affects RAGE glycosylation and thereby affects ligand binding. WT or various mutant forms of RAGE protein, including N25Q, N81Q, N25Q/G82S, and N25Q/N81Q, were produced by transfecting HEK293 cells. The glycosylation patterns of expressed proteins were compared. Enzymatic deglycosylation showed that WT RAGE and the G82S polymorphic variant are glycosylated to the same extent. Our data also revealed N-linked glycosylation of N25Q and N81Q mutants, suggesting that both Asn25 and Asn81 can be utilized for N-linked glycosylation. Using mass spectrometry analysis, we found that Asn81 may or may not be glycosylated in WT RAGE, whereas in G82S RAGE, Asn81 is always glycosylated. Furthermore, RAGE binding to S100B ligand is affected by Asn81 glycosylation, with consequences for NF-κB activation. Therefore, the G82S polymorphism promotes N-linked glycosylation of Asn81, which has implications for the structure of the ligand binding region of RAGE and might explain the enhanced function associated with the G82S polymorphic RAGE variant.

The receptor for advanced glycation end products (RAGE) is a multiligand receptor that binds to carboxymethyl lysine- and AGE-modified proteins and lipids but also to more autonomic ligands including high mobility group box 1 protein (HMGB1), members of the S100/Calgranulin protein family, amyloid β-peptide and Mac-1 (1–7). Binding of ligands to RAGE is mediated by an extracellular region of the receptor comprising an N-terminal variable (V)-domain and two constant (C)-immunoglobulin domains (2, 8, 9). Interaction between RAGE and ligands recruits diverse signal transduction pathways involving NF-κB and MAP kinase activation and consequently the expression of proinflammatory genes (10–14). RAGE also acts as an endothelial adhesion receptor promoting leukocyte recruitment during inflammation via a direct interaction with the β2-integrins Mac-1 or p150,95 expressed by leukocytes (15, 16).

RAGE contains two potential N-linked glycosylation sites at Asn25 and Asn81 (1). It is now well established that RAGE is N-linked glycosylated and that some of the added N-glycan is further modified, resulting in an anionic nonsialylated carboxylated N-glycan (17–20). The nonsialylated carboxylated N-glycan is essential for binding of RAGE to HMGB1, S100A8/A9, and S100A12 in particular, indicating that N-linked glycosylation and/or the modification of the added N-glycan play important roles in the regulation of RAGE-ligand binding (18, 21, 22).

A naturally occurring polymorphism has been identified that results in a glycine-to-serine substitution at position 82 within the V-domain. This polymorphism occurs with relatively high incidence compared with other RAGE polymorphisms that have been identified (23, 24). The G82S mutant RAGE displays enhanced ligand binding to S100A12 and AGE ligands (25, 26). Consequently, this RAGE variant is associated with increased NF-κB activation and inflammatory gene expression (25, 26). In addition, the G82S polymorphism is associated with reduced levels of soluble RAGE (sRAGE) that in a number of diseases magnifies the contribution from RAGE toward inflammation (27–31). How ligand binding and sRAGE levels are altered by the G82S polymorphism is unknown. The G82S substitution occurs within one of the potential N-linked glycosylation consensus sites, involving Asn81. On this basis, we hypothesized that the G82S substitution may influence the glycosylation pattern of RAGE, with consequences for ligand binding and proinflammatory signaling. Here, we describe detailed analysis of the glycosylation of RAGE and identify enhanced glycosylation induced by the G82S polymorphism.
EXPERIMENTAL PROCEDURES

Vector Construction and in Vitro Mutagenesis—Human full-length WT RAGE cloned into pcDNA3.1 (pcDNA3.1/RAGE) was a generous gift from Dr. Ann Marie Schmidt (Columbia University, New York). The predicted N-linked glycosylation sites at Asn²⁵ and Asn⁸¹ within RAGE sequence were separately eliminated by mutation of the Asn residues to Gln (N25Q or N81Q, respectively) using a QuikChange site-directed mutagenesis kit (Stratagene). The site-directed mutagenesis primers were created as follows with mutated residues underlined: N25Q-sense (5’-GTAAGTGTGCTCAAAGATCAC-AAGCCCGAATTTGCCGAG-3’) and N25Q-antisense (5’-CTG-GCCAACTCGGGCTGTCGTCTGTTGAGCACCTAC-3’) or N81Q-sense (5’-GTCGCTGTCTTCCCAAGGCTCTCT-TCTCC-3’) and N81Q-antisense (5’-GGAAGAGGGAGCCCT-TGGGGAAGGCACGACG-3’). Similarly, a Gly to Ser substitution at position 82 (G82S) was introduced using primers designated as G82S-sense (5’-CGTGTCTTCCCAACAGCT-CCCTCTTCTCTCC-3’) and G82S-antisense (5’-GGAAGG-AAGGGGAGCTGTTGGGAAGGCACG-3’). In addition, combination mutants containing both N25Q and G82S substitutions or both N25Q and N81Q substitutions were produced using N25Q-sRAGE cDNA as template and G82S-sense and G82S-antisense primers. All mutations were verified by sequence analysis of the entire plasmid insert.

Cell Culture, Transfection—HEK293 cells were cultured in DMEM (Invitrogen) containing 10% FCS, 50 μg/ml gentamicin (Invitrogen), and 100 units/ml penicillin/streptomycin (Invitrogen; DMEM-10% FCS). Cells were maintained at 37 °C in a 5% CO₂ atmosphere. FuGENE 6 reagent (Invitrogen) was used to transfect HEK293 cells with plasmid cDNA encoding WT or mutant variant forms of RAGE. Whole cell lysates were prepared by solubilization of transient transfected cells in cell lysis buffer containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and 1 × Complete protease mixture (Roche Applied Science) for 1 h. Cell debris was removed using centrifugation at 10,000 × g for 10 min at 4 °C, and the supernatant was used for analysis.

Biotinylation of Cell Surface Proteins—Transient transfected cells expressing WT or mutant forms of RAGE were washed twice with cold PBS and then incubated with PBS containing 1 mg/ml sulfo-NHS-LC-Biotin (Pierce) for 30 min on ice for cell surface biotinylation. The cells were then washed three times with cold PBS and lysed in PBS containing 1% (vol/vol) SDS and 1 × Complete protease mixture to which was added an equal volume of TBS containing 0.4% (vol/vol) Triton X-100. Cell debris was removed by centrifugation at 10,000 × g for 10 min at 4 °C, and the supernatant was used for NeutraAvidin™ protein beads (Pierce) at 4 °C overnight. After three washes with cold PBS, biotinylated cell surface proteins were eluted by boiling for 5 min in SDS-PAGE sample buffer (150 mM Tris, pH 6.8, 5% SDS, 0.08% bromphenol blue, 25% glycerol with 0.01% (vol/vol) 2-mercaptoethanol) and analyzed by Western blotting. As a control, Western blot analysis of the endogenous intracellular protein, Murr-1 was used to confirm that biotinylated RAGE proteins are from the cell surface.

Enzymatic Deglycosylation—Whole cell lysates from transient transfected HEK293 cells were digested with peptide-N-glycosidase-F (PNGase-F; Roche Applied Science). From 10 to 20 μg of protein in 20 mM Tris-HCl, pH 7.5, 120 mM NaCl was denatured by addition of SDS to 0.25% then incubated in a boiling water bath for 10 min. Upon cooling, 2 units of PNGase-F in 100 mM phosphate buffer, pH 7.2, containing 25 mM EDTA and 2.5% (vol/vol) Triton X-100 was added and the samples incubated at 37 °C for 6 h. Duplicate aliquots of whole cell lysates were digested with endoglycosidase-H (endo-H; Roche Applied Science). Protein was denatured in 0.1 M 2-mercaptoethanol containing 0.1% (vol/vol) SDS at 90 °C for 5 min, cooled, and then diluted 1:1.5 in 0.5 mM sodium citrate, pH 5.5, containing 1 × Complete protease inhibitor mixture before adding 1 unit of endo-H and incubation at 37 °C for 6 h. All glycosidase-treated samples were separated by SDS-PAGE and analyzed by Western blotting or mass spectrometry as described below.

Western Blotting—From 5 to 15 μg of cell lysates were analyzed by 12% SDS-PAGE under reducing conditions (32) and Western blotting. RAGE protein was detected using polyclonal goat anti-human RAGE antibody (0.2 μg/ml; R&D Systems) and then with appropriate HRP-conjugated rabbit anti-goat antibody (0.2 μg/ml; Rockland) and Supersignal CL-HRP (Pierce).

Flow Cytometry—Cell surface expression of WT RAGE and mutant RAGE was verified by flow cytometry analysis. Transient transfected HEK293 cells were washed with cold PBS and detached from the cell culture flask by gentle scraping. Cells were then resuspended at 2 × 10⁶ cells/ml in PBS containing 1% BSA. An aliquot of 10⁵ cells was stained with or without a goat anti-human RAGE antibody (25 μg/ml; R&D Systems) and incubated on ice for 30 min. Cells were washed twice by resuspension in PBS containing 0.2% BSA, followed by centrifugation at 200 × g for 3 min. Bound antibody was detected by incubation on ice for 30 min with 100 μl of Alexa Fluor 488 rabbit anti-goat IgG (17.5 μg/ml; Invitrogen). Cells were washed twice and analyzed using a FACScalibur flow cytometer (Becton Dickinson) operated with CELLQuest Pro™ software (Becton Dickinson). For each sample, information on least 5000 cells was collected.

Immunoprecipitation—WT and G82S mutant RAGE were isolated from whole cell lysates using immunoprecipitation. Biotinylated polyclonal goat anti-human RAGE antibody (R&D Systems) was bound to NeutraAvidin™ protein beads (Pierce) by incubating at 4 °C overnight. Whole cell lysate was precleared by incubating with NeutraAvidin™ protein beads (Pierce) at 4 °C for 4 h. RAGE protein was then isolated from precleared lysate by incubation with the anti-RAGE antibody bound to the beads at 4 °C overnight. After three washes with cold PBS, bound RAGE protein was eluted by boiling for 10 min in SDS-PAGE sample buffer.

Mass Spectrometry—RAGE protein eluted following immunoprecipitation was separated on 12% polyacrylamide gel then stained with SimplyBlue™ Safestain reagent (Invitrogen). RAGE protein bands with appropriate molecular mass were excised from the gel and subjected to in-gel tryptic digestion according to Shevchenko (33). Eluted peptides were dried using
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A centrifugal vacuum concentrator and resuspended in 2% (vol/vol) acetonitrile and 0.2% (vol/vol) formic acid in water. All samples were analyzed by HPLC-coupled nanospray tandem mass spectrometry (MS/MS) using an Ultimate 3000 nanoflow HPLC system (Dionex) in-line coupled to the nanospray source of a LTQ Orbitrap XL mass spectrometer (Thermo Scientific). Five μl of peptide solution was loaded onto an in-house packed emitter tip column (75-μm inner diameter fused silica PicoTip (New Objectives) packed with C-18 material (5-μm beads, 100 Å pore size) on a length of 8–9 cm) and separated by an acetonitrile gradient developed from 2% (vol/vol) acetonitrile and 0.2% (vol/vol) formic acid in water to 55% (vol/vol) acetonitrile and 0.2% (vol/vol) formic acid in water over 15 min. Full mass spectra were acquired in the Orbitrap analyzer at full width at half-maximum resolution of 60,000 in profile mode followed by the acquisition of four data-dependent collision induced dissociation MS/MS spectra in the LTQ ion trap allowing two microscans per fragment spectrum. For protein identification MS/MS spectra were searched against a subset of the NCBI nonredundant sequence data base containing all human amino acid sequences (download Nov. 2009; containing 508,360 entries) using the Mascot search engine. All searches were performed for tryptic and nontryptic cleavages and with mass tolerances of 10 ppm for MS and 0.8 Da for MS/MS. The significance threshold for peptide identification by MS/MS data was p < 0.01. Extracted ion chromatograms were plotted using the Xcalibur software. The whole isolope cluster of precursors of interest was selected to account for overlapping intensities of isotope peaks of the unmodified and deamidated peptides.

NF-κB Nuclear Translocation Assay—106 HEK293 cells were transfected with control pcDNA3.1 plasmid (mock) or constructs encoding WT or various mutant forms of RAGE in serum-free DMEM for 6 h. Plasmid DNA was removed and incubation continued in serum-free DMEM for a further 18 h before addition of S100B in PBS to 10 μg/ml. Controls received an equivalent volume of PBS. After 30 min, cells were lysed in 140 μl of 10 mm HEPES, pH 7.9, containing 10 mm KCl, 0.1 mm EDTA, 0.5 mm DTT, 0.2% (vol/vol) Nonidet P-40, and 1 × Complete protease inhibitor mixture for 15 min on ice, then Dounce-homogenized. Nuclei were pelleted by centrifugation at 10,000 × g for 5 min at 4 °C, and the cytosolic supernatant was retained. Nuclei were washed with PBS containing protease inhibitor, then lysed in 25 μl of 20 mm HEPES, pH 7.9, containing 400 mm NaCl, 1 mm EDTA, 10% (vol/vol) glycerol, and protease inhibitor by brief sonication. The nuclear extract was centrifuged at 10,000 × g for 8 min at 4 °C and then analyzed by SDS-PAGE and Western blotting. Western blots were developed using rabbit anti-human NF-κB antibody (1:1500; Sigma) and HRP-conjugated goat anti-rabbit secondary antibody (DAKO) and Superniels CL-HRP (Pierce). Analysis included detection of actin or tubulin proteins using protein-specific mouse antibodies (both Sigma) and HRP-conjugated rabbit anti-mouse secondary antibody (DAKO). Intensities of NF-κB p65 protein bands (normalized to actin bands) in nuclear extracts from S100B-treated cells were compared with those for PBS control-treated cells in three separate experiments. An overall mean value was generated and used to compare differences in response to S100B between mock- and RAGE-transfected HEK293 cells using one-way ANOVA. Statistical significance was defined by p < 0.05.

RESULTS

The G82S Mutation Has No Impact on the Extent of Glycosylation of RAGE in HEK293—To compare glycosylation patterns, WT or G82S mutant variant forms of RAGE were expressed from transient transfected HEK293. The molecular masses of RAGE protein in whole cell lysates were initially established by SDS-PAGE and Western blotting with or without PNGase-F or endo-H treatments (Fig. 1A). It was revealed that HEK293 cells express three species of WT and G82S RAGE with molecular masses of 59, 57, and 52 kDa. After PNGase-F treatment of WT and G82S RAGE, bands of 52 and 50 kDa appeared at the expense of the 59-kDa and 57-kDa proteins expressed by transiently transfected HEK293 cells. A, cell lysates containing WT or G82S RAGE without treatment (−) or after PNGase-F or endo-H treatment (+) were subjected to Western blotting, then detected with RAGE-specific antibody. Untreated WT and G82S RAGE preparations comprise three protein species with molecular masses of 59, 57, and 52 kDa. Following PNGase-F treatment, 52-kDa and 50-kDa protein species were detected in WT and G82S RAGE preparations. A PNGase-F-resistant protein band with a molecular mass of 57 kDa was observed only in WT RAGE preparation. Endo-H treatment revealed 57-, 52-, and 50-kDa protein species in both WT and G82S RAGE preparations. B, cell surface biotinylation of RAGE-expressing HEK293 cells revealed that all of the WT and G82S RAGE protein species were expressed on the cell surface. Cell surface proteins were biotinylated and membrane fractions precipitated with NeutrAvidin beads before detection by Western blotting using anti-human RAGE antibody. Endogenous, intracellular protein Murr-1 was detected only in cell lysate preparations. C, cell surface expression of WT and G82S RAGE on HEK293 cells was confirmed by flow cytometry analysis using RAGE-specific antibody and Alexa Fluor 488 rabbit anti-goat antibody.
The data suggest that WT and G82S RAGE are both N-linked glycosylated to the same extent. The one point of difference in the glycosylation patterns was that for WT RAGE, some of the 57-kDa protein species remained PNGase-F-resistant. This did not occur in G82S RAGE. After separate treatment with endo-H, protein species with molecular masses of 57, 52, and 50 kDa remained in both WT and G82S RAGE preparations (Fig. 1A). Thus, similarities in glycosylation patterns between the two forms of RAGE extend to the type of glycans involved. In each, the 59-kDa protein species is modified by high mannose and/or hybrid glycans, and the 57-kDa protein species is modified by complex glycans.

RAGE is a cell surface receptor. Accordingly, we compared the protein species comprising WT and G82S RAGE, expressed on the cell surface, by using a cell surface biotinylation assay. This showed that all of the protein species from both WT and G82S RAGE are expressed on the cell surface of HEK293 cells (Fig. 1B). Intracellular Murr-1 protein was not biotinylated but could be detected in cell lysate preparations, confirming that biotinylated RAGE protein species are derived from the cell membrane and are not contaminated by cytosolic proteins. Furthermore, flow cytometry analysis confirmed that both WT and G82S RAGE are expressed on the surface of transfected HEK293 cells (Fig. 1C).

Mutational Analysis Suggests That the G82S Polymorphism Promotes N-Linked Glycosylation at Asn81—To investigate further how RAGE is glycosylated, we produced mutant forms of RAGE where Asn25 and/or Asn81 was substituted by Gln, thereby eliminating potential N-linked glycosylation sites. In addition, the G82S mutation was introduced in combination with an N25Q mutation. Surface expression of these mutant forms of RAGE was again confirmed by flow cytometry analysis (data not shown). Moreover, cell surface biotinylation assays showed that all of the protein species comprising mutant forms of RAGE were expressed on the cell surface, indicating that N-linked glycosylation is not necessary for cell surface trafficking of RAGE (Fig. 2). The molecular masses of protein species comprising WT and mutant forms of RAGE are summarized in Table 1. The N25Q/N81Q mutant, which does not contain any N-linked glycosylation sites, comprises two protein species with molecular masses of 52 and 50 kDa. This infers an additional (post-translational) modification to RAGE, which adds ~2 kDa to the molecular mass of RAGE. In the N25Q mutant, an additional species of 57 kDa is present, as well as the 52- and 50-kDa species. This suggests that N-linked glycosylation can occur at Asn81. It has been previously shown that Asn25 is favored for N-linked glycosylation in WT RAGE (34). Combining the data from our mutation analyses suggests that reciprocal promotion of glycosylation occurs in the absence of an alternative glycosylation site. When the Asn81 site is eliminated, the unglycosylated 50-kDa species is absent, suggesting that more efficient N-linked glycosylation of Asn25 occurs. Finally, in the N25Q/G82S mutant, 57- and 52- Da species are present. Comparing component protein species of the N25Q mutant and N25Q/G82S mutant reveals that the unglycosylated 50-kDa species is missing in the N25Q/G82S mutant. This indicates that N-linked glycosylation at Asn81 is promoted by the G82S mutation, when Asn25 is eliminated.

Differences in Asn81 Glycosylation between WT and G82S Mutant RAGE—High resolution HPLC-coupled LTQ Orbitrap mass spectrometry of tryptically digested WT- and G82S RAGE was performed to establish whether N-linked glycosylation of Asn81 is promoted in G82S RAGE. To address this question, the extent of Asn81 to Asp81 conversion (deamidation) was measured after hydrolytic deglycosylation of RAGE by PNGase-F (35–37). To account for nonenzymatic deamidations, we always compared the ratio of tryptic peptides containing deamidated Asn versus the unmodified form before and after PNGase-F treatment. In tryptic digests of WT RAGE, Asn81 was detected in the peptide (VLPN81GSLFLPAVGIQDEGIFR) at m/z 1121.615 of the doubly charged ion. However, a peptide at m/z 1122.107 was also detected that may represent a naturally or nonenzymatically deamidated Asn81 (Fig. 3). Both peptides were chromatographically well resolved with the deamidated form eluting ~15 s later. High mass accuracy Orbitrap measurement and collision-induced dissociation-based peptide fragmentation unambiguously discriminated the unmodified peptide from the deamidated species with significant Mascot ion scores of 101 and 82, respectively (supplemental Fig. 1). Following PNGase-F treatment, the ratio of peak intensities of

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**Table 1**

| WT     | G82S | N25Q | N81Q | N25Q/G82S | N25Q/N81Q |
|--------|------|------|------|-----------|-----------|
| kDa    | kDa  | kDa  | kDa  | kDa       | kDa       |
| 59     | 59   | 57   | 57   | 57        | 57        |
| 52     | 52   | 52   | 52   | 52        | 52        |
| 50     |      |      |      | 50        |           |
| 50     |      |      |      |           |           |
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FIGURE 3. Extracted ion chromatograms of tryptic peptides containing Asn81 of WT and G82S RAGE. WT and G82S RAGE without treatment or after PNGase-F treatment were digested with trypsin and analyzed by nanoflow HPLC-coupled LTQ Orbitrap MS/MS. Peak intensities of the tryptic peptides from WT (VLPN81GSLFLPAVGIQDEGIFR) and G82S (VLPN81SSLFLPAVGIQDEGIFR) RAGE were plotted as extracted ion chromatograms covering the whole isotope envelopes of both the unmodified (Asn81) and deamidated (Asp81) peptide species. The unmodified peptide is chromatographically separated from the deamidated species with the latter eluting approximately 15 s later. Deglycosylation by PNGase-F increases peak intensities of the deamidated versus unmodified species of the peptides. In G82S only the deamidated form could be detected and only after PNGase-F treatment, indicating that the polymorphic variant is fully glycosylated at Asn81.

deamidated (Asp81) versus unmodified (Asn81) peptide was significantly increased as shown by the extracted ion chromatograms of signals between m/z 1121.6 and 1124.2 which covers the isotope clusters of both peptide species (Fig. 3). The increase of deamidation by PNGase-F treatment of WT RAGE indicates that a subpopulation of Asn81 is glycosylated, as recently reported (22).

The situation for WT RAGE contrasts with data from G82S RAGE, where neither the tryptic peptide (VLPN81GSLFLPAVGIQDEGIFR) nor its deamidated form was detected at the expected m/z values of peptide ions. However, prior PNGase-F treatment revealed a strong signal for the deamidated form of the peptide at m/z 1137.117 of the doubly charge species. The deamidated peptide was identified significantly by a Mascot search, resulting in an ion score of 95 (supplemental Fig. 2). No unmodified peptide was detected in the PNGase-F treated G82S RAGE protein. These data confirm that the Asn81 site is fully glycosylated in G82S RAGE with significant amounts of N-linked glycan. Our data clearly show that the G82S polymorphism promotes N-linked glycosylation at Asn81.

Consistent with N-linked glycosylation, Asn25 was not covered by any of the detected peptide species from WT or G82S RAGE. However, following PNGase-F treatment only low intensity MS signals for the deamidated peptide (AQN25TAR) at m/z 387.708 of the doubly charged ion were detected, with Mascot ion scores <44 for MS/MS spectra (data not shown). Accordingly, MS evidence for Asn25 glycosylation in WT and G82S RAGE was inconclusive.

N-Linked Glycosylation Influences the Outcome of RAGE-S100B Ligand Interaction—Asn81 is located in close proximity to a hydrophobic cavity in the RAGE V-domain. Hydrophobic interactions involving residues within this cavity are critical for RAGE-S100B protein ligand binding (38) and are those most likely to be influenced by Asn81 glycosylation. Any effect of N-linked glycosylation on the outcome of the RAGE-S100B ligand interaction was considered through measures of NF-κB activation. NF-κB p65 protein was present in cytoplasmic extracts from mock-transfected HEK293 cells and those expressing WT and mutant forms of RAGE. These levels of NF-κB p65 were not affected by S100B treatment (supplemental Fig. 3).

Consistent with NF-κB activation, S100B treatment increased the levels of NF-κB p65 in nuclear extracts from transfected HEK293 cells expressing WT or G82S RAGE (Fig. 4). Cells expressing N25Q mutant RAGE showed no significant difference in nuclear NF-κB p65 levels from mock-control cells or between S100B-treated and PBS control-treated cells. In contrast, S100B treatment of cells expressing the N81Q mutant caused NF-κB activation (Fig. 4). These data indicate that N-linked glycosylation of Asn81 is required for S100B binding to WT RAGE. However, S100B treatment also induced NF-κB activation through the N25Q/G82S mutant, whereas this RAGE ligand had no effect when acting through N25Q/N81Q mutant RAGE (Fig. 4). It appears that the glycosylation of Asn81 also affects the hydrophobic interaction(s) between S100B ligand and RAGE.

DISCUSSION

The G82S polymorphism of RAGE has important functional implications because it is associated with enhanced ligand binding, reduced sRAGE production, and consequently an enhanced receptor signaling (25–29). Nevertheless, it is not well understood how this RAGE polymorphism affects ligand binding and soluble RAGE production. There are two potential N-linked glycosylation sites within the ligand binding domain of RAGE, at Asn25 and Asn81. The inclusion of the G82S polymorphism within the Asn81 site suggested to us that this polymorphism might affect RAGE glycosylation.

Previously, it has been shown that Asn25 in WT RAGE is always modified with fully processed N-linked glycan, whereas Asn81 is not favored for N-linked glycosylation (34). More recent analysis indicates that Asn81 is modified (22). Our data concur with the glycosylation at Asn25 in WT RAGE and suggest that this is also the case for G82S RAGE. In particular, an N81Q mutant was N-link glycosylated, and tryptic peptides including Asn25 were not detected from either WT or G82S RAGE by MS. However, we were unable to confirm that a deamidated version of this peptide could be generated by PNGase-F treatment. This anomaly in MS analysis is striking in its similarity between WT and G82S RAGE.

A mutagenesis approach also supported that N-linked glycosylation of Asn81 was possible in WT RAGE. For example, glycosylation at Asn81 occurred when Asn25 was eliminated. In addition, a comparison showed there were similar glycosylated protein species (i.e. 57 and 52 kDa) comprising the N81Q and the N25Q mutants that each retained a single, but different Asn for N-linked glycosylation. However, the presence of an additional nonglycosylated 50-kDa component to the N25Q mutant
suggests that the glycosylation of Asn^81^ is less efficient amid WT RAGE protein sequence. We also utilized a combination of MS analysis and PNGase-F digestion to confirm that Asn^81^ is modified by N-linked glycosylation. Specifically for WT RAGE, our results are consistent with a recent report that described the glycosylation of Asn^81^ to involve variable, partially processed glycan (22). Here also, our results indicate that the variable amount of carbohydrate added to Asn^81^ is not guaranteed in WT RAGE. Tryptic peptides derived from WT RAGE were consistent with the presence of both unmodified and deamidated forms of Asn^81^, while having no effect on Asn^25^ glycosylation.

A comparison with G82S RAGE extended the detail of RAGE glycosylation, suggesting that the glycosylation of Asn^81^ is in fact promoted by the naturally occurring G82S polymorphism. Similar to WT RAGE, the absence of Asn^81^ in the G82S polymorphic RAGE variant allowed N-linked glycosylation at Asn^81^. However, further data indicated that Asn^81^ might provide a key point of difference between the glycosylation patterns of WT and G82S RAGE. For example, a comparison of the N25Q mutant in N25Q/G82S mutant revealed that the additional 50-kDa unmodified protein component of the N25Q mutant is missing in the combination mutant. This provided further evidence for less efficient glycosylation of Asn^81^ within WT RAGE and conversely the promotion of Asn^81^ glycosylation within G82S RAGE. In fact, the overall analysis of G82S RAGE-based mutants showed no evidence of variation in the use of the Asn^81^ glycosylation site. Consequently, in G82S RAGE, glycosylation of Asn^81^ is always seen. Again this was verified using a combination of PNGase-F digestion and MS analysis to identify N-linked glycosylation sites in G82S RAGE. In contrast to WT protein, Asn^81^-containing tryptic peptides derived from G82S RAGE were not detected. However, deamidated Asn^81^ peptides were present following PNGase-F treatment. Combined, our data show that the G82S polymorphism promotes N-linked glycosylation of Asn^81^, while having no effect on Asn^25^ glycosylation. A consequence is that in the G82S polymorphic variant of RAGE, Asn^81^ is always glycosylated.

From our data there appears to be contrasting use of Asn^81^ between WT and the G82S polymorphic variant of RAGE. The published evidence from RAGE produced in bacteria (i.e. lacking N-linked glycosylation) indicates that the G82S polymorphism causes a local change around the mutation site and a more global destabilization of the protein structure, with increased flexibility of the V-domain (39). Thus, substitution of Gly^82^ by Ser in G82S RAGE appears to “relax” protein conformation, thereby promoting Asn^81^ glycosylation. A key question is how N-linked glycosylation of Asn^81^ might translate into the increase in ligand binding and decrease in sRAGE production associated with G82S RAGE.
Some insight can be gained from the structure of the ligand binding domain of RAGE (40, 41). The extracellular domain of RAGE contains three immunoglobulin-like domains, including a variable V-domain followed by two constant C-domains, each with a set of conserved cysteine residues (1, 42). In the solved structure the V-domain and first C-domain of RAGE form a single structural unit, whereas the second C-domain moves independently from the V- and C-domain unit (40). Furthermore, a hydrophobic cavity (Ile26, Ala28, Pro33, Leu34, Val35, Leu36, Lys37, Leu49, Trp61, Val63, Leu64, Trp72, Val75, Val78, Leu79, Pro80, Phe85, Leu86, Pro87, Val89, and Tyr113) within the V-domain structure is bordered by cationic residues (Arg29, Lys37, Lys39, Lys43, Lys44, Arg48, Lys52, Arg98, Lys104, Arg107, Lys110, Arg114, and Arg116) and a flexible region (Thr55–Pro71). The flexible region allows further plasticity within the hydrophobic cavity, thereby promoting hydrostatic interactions with RAGE ligand (38, 41), whereas the cationic residues provide points of electrostatic interaction for ligand binding. In fact, the distribution of the cationic residues determines the extent of their contribution to the electrostatic interaction. Those cationic residues located in proximity of the hydrophobic cavity (Lys37, Lys39, Lys43, Arg48, Lys52, Arg98, and Lys104) appear crucial for ligand binding whereas others, on the opposite side with regard to the cavity, have less impact (41). Consequently, RAGE binding to ligand is dependent on hydrophobic and/or electrostatic interactions. The relative contribution from each is ligand dependent. In the RAGE structure, Asn81 is strategically located adjacent to the cationic region and in close proximity of the hydrophobic cavity. In contrast, Asn25 is located on the opposite side of the RAGE molecule (Fig. 5). The inference is that N-link glycosylated Asn81 could play a sentinel role in RAGE ligand binding, controlling the access and binding of ligand to the hydrophobic cavity.

This possibility was considered in assays of RAGE binding to S100B protein ligand. Of relevance is that RAGE binding to S100B ligand is mediated by hydrophobic interaction(s) and involves residues within the hydrophobic cavity (38). As such, these interactions are those most likely to be influenced by the glycosylation of Asn81. We established any influence of glycosylation on this hydrophobic interaction(s) with measures of RAGE-mediated NF-κB activation. This approach accounted for the signaling capacity of the various mutant forms of RAGE but clearly did not distinguish any form of RAGE that bound S100B without signaling. Nevertheless, these assays establish that S100B can induce NF-κB activation through WT and G82S RAGE and the N81Q mutant. A comparative lack of NF-κB activation via the N25Q mutant highlights that Asn25 glycosylation is required for S100B binding/signaling. Given the position of glycosylated Asn25 in the RAGE structure, this suggests an impact of Asn25 glycosylation on protein structure, a possibility that requires further investigation. Further comparisons that show S100B signals through the N25Q/G82S mutant (but not via the N25Q mutant) suggest that glycosylation of Asn81 can compensate for any lack of Asn25 glycosylation but impacts similarly on hydrophobic interactions between RAGE and S100B ligand. This effect is consistent with the possibility that regions of RAGE involving glycosylated Asn25 and Asn81 interact, as previously suggested (22).
These data extend the known influence of glycosylation on the receptor function of RAGE and begin to unravel reasons for the increased ligand affinity shown by G82S RAGE. Earlier studies have shown that substitution of Asn81 with Asp, mimicking deamidation, results in reduced RAGE binding to AGE-lysosome and AGE-BSA (41). Clearly, insertion of the acidic residue opposes electrostatic attraction of AGE-modified ligands. Some of Asn81 in WT RAGE is naturally deamidated to Asp. Given that Asn81 in G82S RAGE is always glycosylated, deamidation of this residue opposes electrostatic attraction of AGE-modified ligands. Combined with recent evidence that substitution of Asn81 with Asp, mimicking deamidation, results in reduced RAGE binding to AGE-RAGE variant and its contribution to inflammation.

The influence of carbohydrate on RAGE-ligand interaction is complicated by possibilities that the nature of the added carbohydrate is different, depending on the site of glycosylation and/or whether there is subsequent modification of glycan to an anionic, nonsialylated carboxylated form. Both WT and G82S RAGE responded similarly to endo-H and PNGase-F treatment, indicating similar complexity in the carbohydrate added to each protein. For WT RAGE, modification can occur for N-linked glycan added to Asn81 (18, 22, 43). The anionic glycan contains glutamic acid linked to the outer regions of the sugar chain and is thought to promote ligand binding through electrostatic interaction between RAGE and its ligands (17). Some ligands, including HMBG1, S100A8/A9, and S100A12, require this anionic glycan to bind to RAGE (18, 22, 43). It remains to be determined whether modifications to glycan at one or both sites of glycosylation are required for ligand binding and whether similar modification is a feature of the Asn81 glycosylation promoted by the G82S polymorphism.

Our finding that the G82S polymorphism promotes N-linked glycosylation at Asn81 identifies a key difference between the WT and G82S forms of RAGE. Combined with recent published data it appears that this difference offers explanation for the enhanced function associated with the G82S polymorphic RAGE variant and its contribution to inflammation.

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