The Amino-terminal Domains of the Ezrin, Radixin, and Moesin (ERM) Proteins Bind Advanced Glycation End Products, an Interaction That May Play a Role in the Development of Diabetic Complications*

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The presence of advanced glycation end products (AGEs) formed because of hyperglycemia in diabetic patients has been strongly linked to the development of diabetic complications and disturbances in cellular function. In this report, we describe the isolation and identification of novel AGE-binding proteins from diabetic rat kidneys. The proteins were purified by cation exchange and AGE-modified bovine serum albumin (AGE-BSA) affinity chromatography. NH2-terminal and internal sequencing identified the proteins as the NH2-terminal domains of ezrin, radixin, and moesin (ERM proteins). Using BIAcore biosensor analysis, human N-ezrin-(1–324) bound to immobilized AGE-BSA with a $K_D$ of $5.3 \pm 2.1 \times 10^{-7}$ M, whereas full-length ezrin-(1–586) and C-ezrin-(325–586) did not bind. Other glycated proteins, such as AGE-RNase, N'-carboxymethyllysine (CML)-BSA, and glycated human serum albumin isolated from hyperglycemic diabetic sera competed with the immobilized AGE-BSA for binding to N-ezrin, but non-glycated BSA and RNase did not. Thus N-ezrin binds to AGEs in a glycation- and concentration-dependent manner. Phosphorylated ezrin plays a crucial role in cell shape changes, cell attachment, and cell adhesion. The effect of AGE-BSA on ezrin function was studied in a tubulogenesis model in which LLC-PK1 cell tubule formation is dependent on phosphorylated ezrin. Addition of AGE-BSA completely inhibited the ability of the cells to produce tubules. Furthermore, in vitro tyrosine phosphorylation of N-ezrin and ezrin was also inhibited by AGE-BSA. These proteins represent a novel family of intracellular binding molecules for glycated proteins and provide a potential new target for therapeutic intervention in the prevention or treatment of diabetic complications.

Diabetes mellitus is associated with very significant morbidity as a result of long-term complications, including nephropathy, retinopathy, neuropathy, and macrovascular disease. Diabetes is characterized by chronic hyperglycemia, which leads to an acceleration of the Maillard reaction. This is a spontaneous reaction between glucose and proteins, lipids, or nucleic acids, particularly on long-lived proteins such as the collagens (1). The presence of AGEs, many of which are still poorly defined, leads to the formation of a range of advanced glycation end products (AGEs). Although research interest has focused predominantly on the measurement and binding interactions of extracellular AGEs, recent studies have indicated that intracellular AGEs are also extremely important (2).

There is substantial evidence for a link between hyperglycemia/AGEs and the pathogenesis of diabetic complications such as retinopathy, nephropathy, neuropathy, and vasculopathy (3). First, levels of AGEs are correlated with the severity of these complications (4). Second, in the non-diabetic mouse, injection of AGEs prepared in vitro leads to histological changes resembling diabetic nephropathy, including mesangial expansion and glomerulosclerosis (5). Finally, pharmacological inhibitors of AGE-dependent pathways retard diabetic complications. Aminoguanidine and related compounds prevent AGE formation (6) and have been shown in experimental models of diabetes to reduce tissue AGE levels and retard the development of neuropathy, retinopathy, and nephropathy (7–9). Although it is well established that hyperglycemia and the presence of AGEs on proteins contribute to complications, the pathways for this process have not been clearly determined.

Previous studies have shown that AGEs prepared in vitro bind to cultured cells via cell surface receptors or binding proteins (10), resulting in binding and internalization of AGE-receptor complexes and proteolytic processing of the AGE ligand. Binding of AGE ligands to cells can also modulate cell function. For instance, cell activation with increased expression of extracellular matrix proteins, adhesion molecules, cytokines, and growth factors has been described (10). Several AGE-binding proteins have been identified, including RAGE, AGE-R1, AGE-R2, AGE-R3, and macrophage scavenger receptors 1 and 2 (11–14). Whether all these proteins bind AGE-modified proteins in vivo is not yet clear.

We have observed increased binding of AGEs to diabetic rat kidney sections, which are reduced in animals treated with aminoguanidine (15). The major binding site did not appear to be any of the known AGE receptors. These findings led us to undertake the isolation and characterization of novel binding...
sites for AGE-modified proteins present in the diabetic rat kidney. We report the purification from diabetic rat kidneys of three proteins, i.e. truncated forms of ezrin, radixin, and moesin (ERM). Members of the ERM family of proteins are structurally very similar and are present on cell surface protrusions. They link the actin cytoskeleton of the cell to the plasma membrane and are thought to play a role in cell shape change, motility, and adhesion (16). Our studies identify ERM proteins as novel binding proteins for AGEs, and we hypothesize that the interaction between ERMs and AGEs may play a role in the development of diabetic complications.

EXPERIMENTAL PROCEDURES

With the exception of calpain inhibitor I, which was from ICN Pharmaceuti cals, protease inhibitors and actin were purchased from Sigma. Protein concentrations were determined by the DC protein assay (BioRad). Restriction enzymes, DNA grade agarose, and reagents for reverse transcription and PCR were from Promega, Melbourne, Australia. The pProEX-HT prokaryotic expression system was from Invitrogen, and Ni-NTA-agarose was from Qiagen. Oligonucleotides were prepared to PCR grade by Geneworks, Thebarton, Australia.

Preparation of AGE-BSA—AGE-modified bovine serum albumin (AGE-BSA), AGE-RNase, and N'-carboxymethyllysine (CML)-BSA were prepared as described previously (17). The extent of chemical modification of lysine residues was determined using 2,4,6-trinitrobenzenesulfonic acid by the method described (18). The extent of modification for CML-BSA and AGE-RNase was 77% for AGE-BSA and 97% for AGE-BSA, respectively. AGE-BSA iodination was performed using the chloramine T method (19) to a specific activity of ~10 000 cpm/mg protein. 125I-AGE-BSA was separated from unbound 125I with a P6DG desalting gel column (Bio-Rad).

Preparation of AGE-BSA Affinity Column—An AGE-BSA affinity column was prepared by coupling AGE-BSA (5 mg/ml) to Aff-Gel 15 according to the manufacturer’s instructions (Bio-Rad). The concentration of ligand bound was ~5 mg/ml resin, and a 1.7 ml column was prepared.

Purification of AGE-BSA-binding Proteins from Diabetic Kidneys—Kidneys from streptozotocin-induced diabetic Sprague-Dawley rats (11.4 g total) were homogenized in solubilization buffer (5 mM/tissue of 20 mM Tris, pH 7.4, containing 100 mM NaCl, 1% Triton X-100, 1 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 units/ml Trasylol) for 2 × 10 s at 18,000 rpm with a Dounce homogenizer and mixed end-to-end for 16 h at 4 °C. Insoluble material was removed by centrifugation at 45,000 × g for 30 min at 4 °C. The supernatant (soluble kidney extract) was collected and dialyzed against 20 mM Tris buffer, pH 7.4, containing 50 mM NaCl and 0.1% Triton X-100 for 16 h at 4 °C.

Fresh protease inhibitors were added, and the soluble kidney extract (45 ml) was applied to a BioRx-70 cation-exchange column (55 ml) at a flow rate of 0.5 ml/min. The column was washed extensively with 20 mM Tris buffer, pH 7.4, containing 50 mM NaCl and 0.1% Triton X-100 until the absorbance at 280 nm was less than 0.02. Bound protein was eluted with a salt gradient from 200 ml to 1 M NaCl in 20 mM Tris buffer, pH 7.4, containing 0.1% Triton X-100. Fractions were analyzed for AGE-BSA binding activity by blotting (see below).

The active fractions were pooled and dialyzed against 20 mM Tris, pH 7.4, containing 50 mM NaCl, 0.1% Triton X-100, and protease inhibitors and analyzed to the AGE-BSA affinity column. The column was extensively washed and the proteins eluted with a salt gradient. Fractions (1 ml) were collected, and absorbance at 280 nm, salt concentration (osmolality), and AGE-BSA binding activity were measured.

SDS-PAGE—Purified AGE-BSA binding proteins were analyzed by non-reducing SDS-12.5% PAGE according to the method of Laemmli (20). Proteins were visualized with Coomassie Brilliant Blue R, molecular masses were estimated from semilogarithmic plots of the migration of standard proteins under reducing conditions run simultaneously.

Ligand and Slot Blotting—Proteins in gels were transferred to nitrocellulose membranes (Osmonics, Westborough, MA) at 20 V for 16 h at 4 °C. Non-specific binding sites were blocked by a 1 h incubation with 20 mM Tris, pH 7.4, containing 50 mM NaCl, 0.1% Tween, and 1% BSA (blocking buffer). The nitrocellulose membrane was probed with 125I-AGE-BSA (1 × 106 cpm/ml) for 2 h at room temperature in blocking buffer in the presence or absence of unlabeled AGE-BSA. The membrane was washed thoroughly and exposed to Kodak BioMax MS film.

Slot blotting was performed similarly to ligand blotting, except the proteins were immobilized directly onto nitrocellulose membranes under vacuum and incubated with 125I-AGE-BSA (1 × 106 cpm/ml) for 2 h at room temperature in blocking buffer in the presence or absence of unlabeled AGE-BSA. Individual slots were counted in a γ-counter for quantitation of bound radioactivity.

Western Blotting—Proteins in non-reducing SDS-PAGE sample buffer were separated by SDS-12.5% PAGE and transferred to nitrocellulose membranes. Membranes were probed with ezrin monoclonal antibody (BD Transduction Laboratories). Reactive proteins were visualized using Super Signal chemiluminescent substrate (Pierce).

Preparative Electrophoresis for Amino Acid Sequencing—AGE-binding fractions eluted from the AGE-BSA affinity column were pooled and concentrated 5-fold (Centrivex, Millipore). Protein (50 μg) was incubated at 37 °C for 15 min in reducing buffer (0.25 mM Tris-HCl, pH 6.8, 1% SDS, glycerol, 0.015% bromphenol blue, and 0.2% 2-mercaptoethanol), subjected to SDS-12.5% PAGE, transferred to a polyvinylidene difluoride membrane for 16 h at 20 V at 4 °C, and stained with 0.025% Coomassie Brilliant Blue R in 40% methanol. Bands were excised and sequenced as described above, using low molecular mass markers to identify the desired bands in the 5–10 KDa range.

Cloning of ERM Constructs—Total RNA was extracted from LIM 2405 human colon cancer cells and reverse transcribed using Super Script II reverse transcriptase (Invitrogen). The resultant cDNA mix was used as template for PCR using Pfu DNA polymerase to prepare cDNAs for ezrin (1–1758 bp), N-ezrin (1–971 bp), and C-ezrin (952–1758 bp). Primers additionally encoded a 5' EcoRI site and a 3' HindIII recognition site. The PCR products were digested with EcoRI and HindIII, isolated from 1% agarose gels, and cloned into the corresponding restriction sites in the expression vector pProEXHT, to express the protein fused to an amino-terminal six-histidine sequence (His6). DNA sequencing confirmed the correct sequences of the inserts.

Expression and Purification of Recombinant ERM Proteins—Competent Escherichia coli (JM109) was transformed with the ezrin, N-ezrin, or C-ezrin expression vectors, and expression was induced with 100 μM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 h at 37 °C. Each recombinant protein was purified by Ni-NTA chromatography. Cleared cell lysate was prepared as described (Qiagen) and applied to a 1 ml Ni-NTA-agarose column, washed thoroughly with high salt wash buffer (200 mM Tris, pH 8.0, containing 300 mM NaCl, 1 mM imidazole, and inhibitors (phenylmethylsulfonyl fluoride, calpain inhibitor 1, leupeptin, and Trasylol)) and also with low salt buffer (20 mM Tris, pH 8.0, containing 150 mM NaCl, 0.1% Tween, 20 mM imidazole, and inhibitors) before eluting with low salt buffer containing 250 mM imidazole. Isolated recombinant proteins were dialyzed to remove imidazole prior to further studies.

Preparation of Human Serum Albumin (HSA) from Diabetic Patient Serum—HSA was isolated from sera of two hyperglycemic patients with HbA1C values >9.5% (normal ≤ 6%) using a 1 ml Aff-Gel Blue affinity column. HSA was eluted with 1 M NaCl, concentrated, and dialyzed against Hepes-buffered saline (10 mM Hepes, pH 7.4, containing 150 mM NaCl, 0.1% Tween, 20 mM imidazole, and 0.005% P20) before biosensor experiments.

Biacore Biosensor Analysis—AGE-BSA binding studies were carried out on each of the isolated recombinant full-length and truncated ezrin proteins using surface plasmon resonance on a Biacore 2000 biosensor (Biacore AB).

A CM5 research sensor chip was activated with N-ethyl-N-(3-dimethylaminopropyl)carbodiimidehydrochloride/N-hydroxysuccinimide (EDC/NHS), and AGE-BSA was injected in coupling buffer over the active surface until a suitable resonance unit (RU = 650) value was obtained (1 RU equals to 1 pg of protein bound/mm2 of sensor surface). Remaining activated groups were deactivated by injection of ethanolamine (20 mM) to subtract background binding, and specific binding to glycated moieties in AGE-BSA. Ezrin, N-ezrin, and C-ezrin (150 nm–2.4 μM in Hepes-buffered saline) were passed over immobilized AGE-BSA for 240 s at a flow rate of 10 μl/min. The infusion was stopped and dissociation was observed over 200 s, during which time Hepes-

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Fig. 1. Isolation and characterization of rat kidney AGE-BSA binding proteins. A, detergent extract from diabetic kidneys was subjected to cation exchange chromatography on BioRex-70 column and eluted with a NaCl gradient (solid line). B, the active material (fractions 21–52) was applied to an AGE-BSA affinity column and eluted with a NaCl gradient (solid line). Absorbance (280 nm) values (square) and specific AGE-BSA binding activity (circle) are plotted for every fifth fraction. C, Coomassie Blue staining of the peak fraction (fraction 19) eluted from the AGE-BSA column. The migration of molecular mass (MW) markers is shown.

buffered saline alone was passed over the chip. The binding curves were analyzed using BIA Evaluation 3.0 software. For competition experiments, mixtures of proteins were applied to the chip. In these experiments, varying concentrations of glycated proteins (AGE-BSA, HSA isolated from diabetic sera, AGE-RNase, and CML-BSA) and non-glycated proteins (BSA, RNase, or C-ezrin) were mixed with N-ezrin for at least 30 min before passing over the chip. In other experiments, insulin-like growth factor binding protein 6, (IGFBP-6) (21), an unrelated protein of similar size to the truncated ezrin proteins, was used as a further control for possible nonspecific binding or steric hindrance effects.

Tubulogenesis—Cells from the porcine kidney epithelial cell line LLC-PK1 were cultured in 24-well plates in DMEM containing 10% FCS. Confluent LLC-PK1 cells were stimulated to form tubules by the addition of DMEM/10% FCS containing 250 μg/ml of AGE-BSA, HSA isolated from diabetic sera, AGE-RNase, and CML-BSA) and non-glycated proteins (BSA, RNase, or C-ezrin) were mixed with N-ezrin for at least 30 min before passing over the chip. In other experiments, insulin-like growth factor binding protein 6, (IGFBP-6) (21), an unrelated protein of similar size to the truncated ezrin proteins, was used as a further control for possible nonspecific binding or steric hindrance effects.

To identify the purified AGE-BSA binding-proteins, amino-terminal amino acid sequencing was performed on each of the three bands. The amino acid sequences were PK(T/P)I(S/T)NPQQPK and PK(T/P)I[S/T]NRQPK, which match the sequences up to amino acid 318 of the rat moesin sequence and up to amino acid 321 of the mouse ezrin/radixin sequence. (The most COOH-terminal sequences identified were KAQAREEKHLQKQLER, KAQAREVHLQKQLER, and KAQAREEKH-
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QKQ). However, no evidence was obtained for the presence of the next CNBr fragment (aa 319/322 onwards). Thus, the isolated proteins that bind AGE-BSA are the NH2-terminal 1–320 amino acids of the ERM proteins, which is consistent with the size of the proteins seen by SDS-PAGE.

To define regions of ERM proteins involved in binding AGEs, the human cDNA for ezrin was cloned into the expression vector pPROEX-HTb. Three constructs with NH2-terminal His6 tags were produced, i.e. full-length ezrin (aa 1–586), N-ezrin (aa 1–324), and C-ezrin (aa 323–586). All three constructs were expressed in E. coli and purified by Ni-NTA-agarose chromatography.

The isolated recombinant proteins were characterized by SDS-PAGE (Fig. 3A), Western blotting with an anti-ezrin antibody, which recognizes an epitope in the aa 395–515 region of ezrin (B), and ligand blots (C) with 125I-AGE-BSA (2.5 nM) of N-ezrin (lanes i), ezrin (lanes ii), and C-ezrin (lanes iii) are shown.

Fig. 3. The AGE-BSA binding site is located in the NH2-terminal region of ezrin. Coomassie stain (A), Western blot probed with a monoclonal anti-ezrin antibody, which recognizes an epitope in the aa 395–515 region of ezrin (B), and ligand blots (C) with 125I-AGE-BSA (2.5 nM) of N-ezrin (lanes i), ezrin (lanes ii), and C-ezrin (lanes iii) are shown.

Binding of N-ezrin at different concentrations (0.15, 0.3, 0.45, 0.9, 1.2, and 2.4 μM, represented by the curves from bottom to top, respectively) to immobilized AGE-BSA using BIAcore surface plasmon resonance. RU, resonance unit.

Fig. 4. Concentration-dependent binding of N-ezrin to AGE-BSA. Binding of N-ezrin at different concentrations (0.15, 0.3, 0.45, 0.9, 1.2, and 2.4 μM, represented by the curves from bottom to top, respectively) to immobilized AGE-BSA using BIAcore surface plasmon resonance. RU, resonance unit.

We have identified a novel interaction between AGE-modified proteins and the NH2-terminal regions of the ERM proteins (ezrin, radixin, and moesin), which belong to the erythrocyte protein 4.1 superfamily. The best-known function of ERM proteins within the cell is as a linker between the cytoplasmic tail of membrane proteins and cytoplasmic actin filaments (16). Additionally, ERM proteins have other functions, including regulation of Rho kinase and other signaling molecules such as focal adhesion kinase (FAK) and phosphatidylinositol 3-kinase (PI3K) and modulation of membrane ion transport proteins (NHE1 and 3) (26).

Erm proteins have three structural domains as follows: (i) an N-domain (aa 1–320), which is the most highly conserved (>85%) compared with the other members of the superfamily; (ii) an α-helical mid-region; and (iii) a C-domain, which has regions of positively charged amino acids and a consensus sequence for F-actin binding located in the last 35 amino acids (16). Binding studies with recombinant ezrin constructs re-
vealed that the binding site for AGE-BSA was located in the N-domain. Many plasma membrane ligands (such as CD44 (27), CD43, and intercellular adhesion molecule (ICAM) 1, 2 and 3 (28)) bind via their cytoplasmic domains to the N-domain of ERM proteins. Recent studies show that phosphatidyl inositol 4-phosphate (PIP), phosphatidyl inositol 4,5-biphosphate (PIP2), Rho GTPase, Rho GDP dissociation inhibitor (GDI) (29), syndecan 2 (30), and the sodium-hydrogen exchanger regulatory factor (NHERF), previously known as ezrin-binding protein 50 or EBP50 (31), also bind to the N-domains of ERM proteins. Besides the F-actin binding site in the C-domain, two additional actin binding sites have also been described in the N-domain, although the physiological relevance of these sites is not yet clear (32).

Like AGE-BSA, ligands such as CD44, NHERF, and the Rho GDP dissociation inhibitor bind to N-ezrin but do not bind to full-length ezrin (33). In full-length ERM proteins, the N- and C-domains interact with the F-actin cytoskeleton, while the N-domain is involved in binding various membrane proteins.

FIG. 5. Inhibition of N-ezrin binding to immobilized AGE-BSA by glycated proteins. A, N-ezrin (1 μM), alone or following preincubation (30 min) with potential competitive inhibitors (10 μM), was injected over immobilized AGE-BSA. N-ezrin + RNase, N-ezrin alone, N-ezrin + BSA, N-ezrin + CML-BSA, N-ezrin + AGE-BSA, N-ezrin + AGE-RNase, BSA alone, and RNase alone are represented by the curves from top to bottom, respectively. B, inhibition of N-ezrin binding to immobilized AGE-BSA by BSA from diabetic serum. N-ezrin (1 μM), alone or following preincubation (30 min) with HSA from diabetic serum or non-glycated BSA, was injected over immobilized AGE-BSA. N-ezrin + BSA (10 μM), N-ezrin alone, and N-ezrin + HSA (10 μM) are represented by the curves from top to bottom, respectively. C, C-ezrin did not bind to immobilized AGE-BSA but inhibited the binding of N-ezrin. N-ezrin (1 μM), alone or following preincubation (30 min) with C-ezrin (1 or 2 μM), was injected over the immobilized AGE-BSA. N-ezrin alone (1 μM), N-ezrin + C-ezrin (1 μM), and N-ezrin + C-ezrin (2 μM), are represented by the curves from top to bottom, respectively. RU, resonance unit.

FIG. 6. In vitro phosphorylation of N-ezrin and ezrin by EGFR is inhibited by AGE-BSA. N-ezrin (2.5 µg) or ezrin (15 µg) was phosphorylated by EGFR (1.62 units) in kinase buffer in the presence of BSA or AGE-BSA (30 μM) for 30 min at 30 °C. The reaction was stopped by the addition of SDS-PAGE sample buffer. A, the resultant phosphorylated N-ezrin and ezrin proteins were detected by Western blotting using an anti-phosphotyrosine antibody conjugated to HRP (α-PY). Results shown are representative of three independent experiments. B, densitometric analysis was performed, and the data shown represent the means ± S.E. of three independent experiments.

FIG. 7. AGE-BSA inhibits tyrosine phosphorylated ezrin-dependent tubulogenesis. Porcine kidney epithelial cells, LLC-PK1, were cultured in DMEM containing 10% FCS, and tubule formation was stimulated with DMEM/10% FCS containing 50% fibroblast 3T3 conditioned medium and collagen 1 (120 µg/ml) + BSA or AGE-BSA (40 μM). Tubules formed were counted at 48 h, and results were expressed as a percentage of tubes formed in the control wells cultured in the absence of BSA or AGE-BSA. Cells grown without the 3T3-conditioned medium did not form tubes. Results are mean ± S.E. of three independent experiments. *, p < 0.05; ***, p < 0.01. The HGF control (100%) represents 33 ± 2.64 tubules/well (mean ± S.E.).
C-domains associate by a high affinity intramolecular interaction that masks the binding sites for these ligands and actin (24). Low-angle shadowing electron microscopy recently reported for radixin revealed a compact globular structure that is changed on activation by phosphorylation to an elongated structure with two globular domains linked by a thin, filamentous region (34). In the experiments presented here, full-length ezrin did not bind to AGE-BSA by ligand blot, surface plasmon resonance, or affinity chromatography (data not shown), suggesting that the AGE-BSA binding site on N-ezrin may also be masked by interaction with C-ezrin. This hypothesis was confirmed by the ability of C-ezrin to block the binding of N-ezrin to AGE-BSA.

Because AGE-BSA only binds to recombinant NH$_2$-terminal ezrin or truncated ERMs isolated from diabetic kidneys, ERM activation or proteolysis would have to occur in vivo for AGE binding. Although every care was taken to inhibit proteolytic degradation of proteins during the isolation (i.e. addition of protease inhibitors and purification at 4 °C), it remains possible that truncated ERMs isolated from diabetic kidney may be products of the isolation procedure. However, both full-length ezrin and truncated ERMs were detected by ezrin immunoblotting in the diabetic kidney extract, whereas recombinant full-length ezrin bound to the cation-exchange column but not to the AGE-BSA affinity column, with no evidence of ezrin proteolysis during the isolation steps (data not shown). There is some evidence that proteolysis and activation of ERMs occurs in vivo (29, 35–38), which would make the proteins accessible for binding to glycated proteins.

When bovine endothelial cell monolayers polarize and crawl in response to injury, ezrin proteolytic breakdown products accumulate. The ezrin degradation was found to follow a transient increase in intracellular calcium and could be blocked by a specific calpain I inhibitor (35). In situ experiments with intact gastric glands treated with calcium ionophore also show rapid hydrolysis of ezrin (36). Activation of platelets with thrombin or calcium ionophore caused rapid proteolysis of platelet ezrin and moesin (37). A primary event of cell activation by external stimuli such as high glucose or AGEs is a rise in intracellular calcium, and chronic hyperglycemia is also associated with a decrease in calcium efflux from cells leading to sustained elevation of basal calcium levels in diabetes (39, 40). These observations, together with the results presented in this paper, suggest that the occurrence of high glucose and the glycated proteins present in patients with diabetes create an intracellular environment with high calcium levels that may lead to proteolyzed ERM proteins capable of interacting with intracellular glycated proteins and cause derangements in cellular function.

Activation of full-length ERM proteins is incompletely understood, but phosphorylation of specific residues, dimerization, or phospholipid (phosphatidyl inositol 4,5-biphosphate in particular) binding may be involved (29, 38, 41). ERM proteins can be phosphorylated in vitro, and two phosphorylation sites (Tyr-145 and Thr-567 in ezrin) are conserved within the three ERM proteins (25, 42). The addition of epidermal growth factor to human epidermoid cancer cells (A-431) leads to tyrosine phosphorylation of ezrin and the recruitment of ezrin and actin to membrane projections (43). Inclusion of a tyrosine kinase inhibitor completely blocks both tyrosine phosphorylation of ezrin and its translocation to the membrane (44).

We investigated whether EGFR-induced ezrin tyrosine phosphorylation was changed by the addition of AGE-BSA and showed that AGE-BSA inhibited tyrosine phosphorylation of N-ezrin by EGFR. The fact that phosphorylation of full-length ezrin by EGFR was partially inhibited by AGE-BSA suggests that, although AGE-BSA does not bind to full-length ezrin, phosphorylation by EGFR may open up the protein to permit binding of AGE-BSA with subsequent inhibition of further phosphorylation.

Several studies have shown that ERM proteins and, in particular, ERM phosphorylation, play a crucial role in cell shape changes, cell attachment, and cell adhesion. Suppressing the expression of all three ERM proteins inhibited cell-cell and cell-substrate adhesion, microvilli disappeared, and collapsed actin fibers were observed (45). In studies where ezrin phosphorylation was reduced (similar to our results with AGE-BSA), normal ezrin-mediated cross-linking between the plasma membrane and the actin cytoskeleton was broken. Instead, ezrin remained in the cytoplasm despite ligand induction, and normal cellular responses such as cell shape change and adhesion/attachment were lost or reduced (44, 46).

Furthermore, two tyrosines of ezrin, which are phosphorylated in response to HGF or EGFR, are essential for cell motility and tubulogenesis (22). Transfection of LLC-PK1 cells with ezrin mutated at Y145F and Y353F inhibited cell migration and tubulogenesis. Rearrangements of the actin cytoskeleton underlie cell motility and the formation of long tubules as shown by blockage with cytochalasin B (22). Because AGE-BSA inhibits both tubulogenesis, for which phosphorylated ezrin is an effector, and in vitro phosphorylation of N-ezrin, we hypothesize that glycated proteins may modulate ERM function by inhibition of ERM phosphorylation.

The significance of ERM protein binding to AGEs in the development of diabetic complications remains to be established. AGE-BSA and AGE-RNase produced in vitro are highly modified molecules (~77% modified), and, thus, may not reflect endogenous AGEs. The degree of protein modification is extremely variable in vivo (determined by ambient hyperglycemia, oxidative stress, protein identity, and turnover), and rates of glycosylation with intracellular sugars such as fructose, glucose-6-phosphate, and glyceraldehyde 3-phosphate are much greater than those achieved extracellularly with glucose (47). Indeed, endogenously glycated HSA isolated from human diabetic sera as well as CML-BSA competed with the immobilized AGE-BSA for binding to N-ezrin. N'-carboxymethyllysine is a modification that occurs in vivo (1).

ERM proteins are present at all sites of diabetic complications, and we expect that AGE binding might inhibit ERM functions involving interactions with the N-domain of ERM proteins. These functions include cross-linking between plasma membrane proteins and the actin cytoskeleton and disruption of signal transduction pathways involving phosphorylated tyrosine residues of ERM proteins and other signaling molecules. Thus, the envisioned result of high levels of glycated proteins on ERM responses would be actin disorganization and disconnection from ligand/receptor stimulation and, consequently, abnormal cell-cell adhesion, cell-extracellular matrix attachment, and irregular cell shape. Indeed, there have been several reports of reorganization or disassembly of actin filaments in response to high glucose or AGEs (48, 49), and actin rearrangement has also been shown in vivo in experimental diabetes (50).

Actin cytoskeleton disassembly is a prominent feature of diabetic nephropathy (48) and is particularly evident and important in the podocyte or glomerular epithelial cells. These cells differentiate into a special structure called the glomerular filtration apparatus, which includes the foot processes and slit diaphragms with highly organized actin bundles. These glomerular epithelial cells create a pathway for the glomerular filtrate (51). Ezrin is concentrated along the apical membrane of the foot processes in association with podocalyxin, NHERF2,
and actin (52, 53), and this complex is essential for proper foot process organization. Disruption of this complex leads to the loss of foot process architecture, as seen in diabetes, with effacement of the slit pores and subsequent increases in glomerular permeability and proteinuria (51, 53, 54).

The cofactor role of ERMs in the regulation of the sodium/hydrogen exchanger 3 (NHE-3) is also relevant to diabetic complications. We have reported previously that inhibitors of AGE formation as well as cariporide (an inhibitor of a sodium/hydrogen exchanger) can attenuate the development of diabetic complications in a rat model (55). It remains to be determined if this relates to a central role of ERM proteins that not only bind AGES but also NHERF 1 and 2 (56) and, thus, modulate sodium/hydrogen exchangers.

ERM proteins are novel intracellular binding proteins for glycated proteins. This interaction may modulate a range of processes involved in the development of diabetic complications. Understanding this interaction may thus provide a new avenue for therapeutic intervention and the development of organ-protective agents in diabetes.

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The Amino-terminal Domains of the Ezrin, Radixin, and Moesin (ERM) Proteins Bind Advanced Glycation End Products, an Interaction That May Play a Role in the Development of Diabetic Complications

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