Identification of Galectin-3 As a High-Affinity Binding Protein for Advanced Glycation End Products (AGE): A New Member of the AGE-Receptor Complex

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ABSTRACT

Background: Advanced glycation end products (AGE), the reactive derivatives of nonenzymatic glucose-protein condensation reactions, are implicated in the multiorgan complications of diabetes and aging. An AGE-specific cellular receptor complex (AGE-R) mediating AGE removal as well as multiple biological responses has been identified. By screening an expression library using antibody against a previously identified component of the AGE-R complex p90, a known partial cDNA clone was isolated with homology to galectin-3, a protein of diverse identity, and member of the galectin family.

Materials and Methods: To explore this unexpected finding, the nature of the interactions between galectin-3 and AGE was studied using intact macrophage-like RAW 264.7 cells, membrane-associated and recombinant galectin-1 through -4, and model AGE-ligands (AGE-BSA, FFI-BSA).

Results: Among the members of this family (galectin-1 through 4), recombinant rat galectin-3 was found to exhibit high-affinity ^{125}I-AGE-BSA binding with saturable kinetics (kD 3.5×10^7 M^{-1}) that was fully blocked by excess unlabeled naturally formed AGE-BSA or synthetic FFI-BSA, but only weakly inhibited by several known galectin-3 ligands, such as lactose. In addition to the p90, immunoprecipitation with anti-galectin-3, followed by ^{125}I-AGE-BSA ligand blot analysis of RAW 264.7 cell extracts, revealed galectin-3 (28 and 32 kD), as well as galectin-3-associated proteins (40 and 50 kD) with AGE-binding activity. Interaction of galectin-3 with AGE-BSA or FFI-BSA resulted in formation of SDS-, and β-mercaptoethanol-insoluble, but hydroxylamine-sensitive high-molecular weight complexes between AGE-ligand, galectin-3, and other membrane components.

Conclusions: The findings point toward a mechanism by which galectin-3 may serve in the assembly of AGE-R components and in the efficient cell surface attachment and endocytosis by macrophages of a heterogenous pool of AGE moieties with diverse affinities, thus contributing to the elimination of these pathogenic substances.

INTRODUCTION

Glucose and other reducing sugars react spontaneously with a wide spectrum of proteins in vitro and in vivo to initiate a post-translational modification process, the late products of which comprise of a heterogeneous group of irreversible adducts called advanced glycation end products (AGE) (1,2). In vivo formation of AGE-proteins proceeds slowly under normal ambient glucose concentrations, while the rate of AGE accumulation is enhanced in the presence of hyperglycemia, as in diabetes mellitus (3,4). Numerous studies suggest that AGEs play an important role in the structural and functional alterations that occur in long-term diabetes and more slowly in normal aging (1–5).

Binding and internalization of AGE-modified proteins is facilitated through specific cell surface

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receptors identified first on cells of the monocyte/macrophage lineage (6,7) and subsequently on endothelial (8), mesangial cells (9) and other cell types (4,10). In addition to the uptake and degradation of senescent AGE-modified proteins by macrophages, AGE-receptor/ligand interactions initiate a range of biologically important cellular responses, including chemotaxis, activation, cytokine, and growth factor secretion (4,5). Based on these properties, cellular AGE-receptors are thought to contribute to normal growth patterns and tissue turnover.

Initial studies to determine the molecular composition of AGE receptors revealed two AGE-binding polypeptides at approximately 30 and 90 kD, based on AGE-specific affinity precipitation of radiolabeled cell surface proteins from the murine macrophage cell line, RAW 264.7 (11,12). Subsequently, two AGE-binding proteins, designated p60 and p90, were isolated from rat liver membranes and partially sequenced (13). Antisera raised against purified p60 and p90 recognized surface determinants and blocked AGE binding and AGE-dependent responses of human monocytes/macrophages (13), rat T cells (14), and murine mesangial cells (15). This broad range of activity across cellular systems and species suggested that this AGE-receptor system involves highly conserved proteins. Additional AGE-binding proteins, a 35-kD protein, named RAGE, and an 80-kD protein homologous to lactoferrin were also described (16,17), further expanding this novel class of molecules. The molecular mechanisms by which single AGE-binding proteins associate with other membrane proteins, how they form complexes with them or how they specifically recognize various members of the highly heterogeneous family of AGE structures is unknown. AGE-receptor ligand interactions are only weakly inhibited by free carbohydrates or by proteins modified by early glycation products such as the Amadori adduct (6,8,9,14).

Other mammalian proteins with lectin-like affinity for a spectrum of sugars and glycoconjugates have been described (18,19). Among them, galectins consist of a well defined family of molecules sharing characteristic amino acid sequences and affinity for β-galactoside sugars (19,20). Of these, galectin-3, first described as a cell surface marker for activated macrophages (Mac-2) (21), was subsequently identified as a lactose-specific lectin present on various cells and cellular compartments (22–25). The cDNA and the protein product have been characterized by several laboratories, reporting it as a 35-kD carbohydrate-binding protein (CBP35), a nuclear protein with a role in cell cycle regulation (22); a 32-kD molecule isolated from rat basophilic leukemia cells that binds rat IgE through carbohydrate moieties (26), and as a 29-kD lung lectin with lactose specificity (HL29) (25). The principal physiological role of this molecule in humans remains unknown.

In this report, we describe the identification of the polypeptide currently termed galectin-3 as a macrophage cell membrane protein that exhibits high-affinity binding for nonenzymatically glycated (AGE)-modified proteins and which facilitates covalent complex formation with these ligands.

MATERIALS AND METHODS

Chemicals and Reagents

Recombinant galectin-3 (rat, and murine CBP-35) was prepared as previously described (26). C-terminal domain peptide (18 kD) of murine galectin-3 was a generous gift from Dr. John L. Wang (Michigan State University, East Lansing, MI, U.S.A.). Recombinant human galectin-1 and -2, and rat galectin-4 (domain I) were provided by Drs. H. Leffler and S. H. Barondes (University of California, San Francisco), bovine serum albumin (BSA) (Fraction V, low endotoxin), Nonidet P-40 (NP-40), and Triton X-100 were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN, U.S.A.). Glucose, lactose, galactose, ovalbumin, hydroxylamine, hydrazine, 2-PAM and glycoconjugates were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium ^{125}I (s.a. 10 mCi/100 μl) was obtained from New England Nuclear (Boston, MA, U.S.A.). Nitrocellulose membranes were from Schliecher & Schuell (Keene, NH, U.S.A.). The chemically synthesized AGE, 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole hexanoicacid (FFI-HA) (27,28) was generously provided by Dr. Peter Ulrich (The Picower Institute, NY, U.S.A.). Pyrraline and pentosidine were a gift of Dr. Vincent Monnier (Case Western Reserve, CL).

Preparation and Radiolabeling of Ligands

AGE-modified bovine serum albumin (AGE-BSA) and ovalbumin (AGE-Ova) were made by incubating each protein with 0.5 M glucose at 37°C for 6 weeks in phosphate-buffered saline

(400 mM, pH 7.4), as previously described (13,15). Unincorporated glucose was removed by dialysis against PBS. AGE-modification was measured by an AGE-specific ELISA (29). AGE-BSA contained approximately 300 AGE U/mg, AGE-OVA 1000 AGE U/mg, and BSA contained <3 U/mg.

The chemically defined model AGE, FFI-HA, was synthesized and linked to BSA as described previously (27). Ligand radioiodination was performed with carrier-free-^{125}I using IODO-beads (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's instructions.

Membrane Preparation

Cells of the murine macrophage-like cell line, RAW 264.7 were cultured in monolayers and were collected by gentle scraping and washing in phosphate buffered saline (PBS), centrifuged at 500 × *g* for 5 min, disrupted with a tight Dounce homogenizer, in a solution of PBS containing 1 mM EDTA and protease inhibitors, 2 mM phenyl-methyl-sulfonyl-fluoride (PMSF), 10 μg/ml aprotinin, 5 ng/ml pepstatin, 1 mM benzamidine. Nuclei and cell debris were removed by centrifugation at 1000 × *g* for 10 min. The cellular membranes were isolated from the supernatant by centrifugation at 10,000 × *g* for 20 min at 4°C. The resulting membrane-enriched fraction was solubilized in PBS containing 0.5% NP-40, and protease inhibitors as described above. This material was then used for ligand and Western blot studies. In addition, whole cell extracts were prepared by lysing cells in PBS containing 0.5% NP-40 and protease inhibitors. After 10 min incubation on ice, nuclei and cell debris were removed by centrifugation at 10,000 × *g* for 10 min. Protein concentrations were determined by BCA protein assay (Pierce).

Ligand and Western Blotting

In most studies described below, pure recombinant murine galectin-3 or cell membrane preparations were mixed with an equal volume of Laemli 2 × SDS-PAGE sample buffer containing 5% 2-mercaptoethanol, electrophoresed on 12% SDS-PAGE, and then electroblotted onto a nitrocellulose filter, as previously described (30). For ligand blot analysis, following blocking for 1 hr in a solution of PBS containing 1.5% BSA and 0.1% Triton X-100, 1 mM $MgCl_2$ and 1 mM $CaCl_2$, the nitrocellulose filters were probed ^{125}I-AGE-BSA (300 nM) in blocking solution in the presence or absence of various competitors. The blots were washed three times with PBS containing 0.1% Triton X-100 and exposed to Kodak XAR-5 film at −80°C. Quantitation of bound radioactivity was performed on a Molecular Dynamics phosphorimager and the values were expressed as relative phosphorimage units. For Western blot analysis, following blocking with PBS containing 2% BSA, electroblotted proteins were probed with various primary antibodies as indicated and visualized by using alkaline phosphatase-conjugated secondary antibodies and the NBT/BCIP western blot detection method (31). In separate studies, recombinant human galectin-1, -2, and -3, and rat galectin-4 (domain I) were subjected to SDS-PAGE through a 4–20% gel (5 μg/lane) and electroblotted onto nitrocellulose membranes. Filters were either stained with Amido black or probed with ^{125}I-AGE-BSA.

Antibodies

Avian anti-rat polyclonal antibodies, raised against purified rat liver AGE-binding proteins p60 and p90, were prepared as described (13). Rat monoclonal antibody (mAb) specific to murine galectin-3 was purified from culture supernatants of hybridoma M3/38 (ATCC TIB166) using protein G-sepharose column (Boehringer-Mannheim Biochemicals). Isotypic rat IgG2a was purchased from Zymed Immunochemicals (South San Francisco, CA, U.S.A.). A polyclonal antibody to CBP-35 was generously provided by Dr. J. L. Wang, Michigan State University.

Radiolabeling, Immuno-Precipitation and Ligand-Precipitation

RAW 264.7 cells (1 × 10^7) were surface radiolabeled using lactoperoxidase-catalyzed iodination as described (30). After surface iodination, detergent-solubilized whole cell extracts were precleared by incubation with BSA-Sepharose for 1 hr at 4°C. For immunoprecipitation, 2.5 μg of mAb M3/38 or the isotype control (rat IgG2a) were added and the incubation was extended for 14 hr at 4°C with gentle rocking. To isolate the Ab-Ag complexes, goat anti-rat antibodies, linked to agarose beads (Sigma) were added to the incubation. After an additional 2 hr, the Ab-Ag complexes were isolated by centrifugation at 2,000 × *g* and washed four times with PBS containing 0.5% NP-40. An equal volume of Laemli SDS-PAGE buffer containing 2.5%

β-mercaptoethanol was added to the obtained pellets. For ligand affinity precipitation, AGE-BSA was coupled to activated Sepharose-4B as described (13). After preclearing the cell extracts with BSA-Sepharose, AGE-BSA-Sepharose was added and the incubation was extended for 1 hr at 4°C with gentle rocking. The complexes were separated by centrifugation at 2000 $\times$ g and washed extensively with PBS containing 0.5% NP-40. An equal volume of Laemli SDS-PAGE buffer containing 2.5% β-mercaptoethanol was added to the washed ligand affinity precipitate. Following boiling for 2 min, the proteins were electrophoresed through a 12% SDS-PAGE gel. Ligand- and immuno-precipitated proteins were visualized by autoradiography.

Aggregation Studies

Recombinant murine galectin-3, M3/38-immunoprecipitated galectin-3 or NP-40 detergent-solubilized cell membrane preparations were mixed with AGE-BSA or FFI-BSA at 1 μg/ml in PBS containing 1 mM $CaCl_2$ in the presence or absence of hydroxylamine (50 mM), hydrazine (100 mM) or 2-PAM (50 mM). The aggregates were visualized by autoradiography of dried gels and the amount was quantitated by phosphorimage analysis.

Immunofluorescence

RAW 264.7 cells were plated on coverslips coated with BSA at 1 mg/ml and were grown in DMEM for 2 days. Prior to assay, the medium was removed and replaced with 1 ml of fresh medium in the presence or absence of 50 mg/ml AGE-BSA. After the incubation, the cells were fixed in 3.5% formalin in PBS for 10 min at room temperature, blocked in PBS containing 0.1% BSA and 0.02% sodium azide for 15 min. Anti-galectin-3 and isotype control mAb were added to the cells and the incubation was allowed to continue for 1 hr. After washing the cells four times in PBS, FITC-conjugated goat anti-rat IgG $(Fab)_2$ was added to the cells and incubated for 1 hr at room temperature. The cells were then washed four times in PBS and photographed.

RESULTS

Antibody to the AGE-Binding Protein p90 Recognizes Galectin-3

By screening an expression library from activated macrophages with an antibody made to the 90-kD subunit (p90) of the AGE receptor complex (13), we isolated a sequence that corresponded to galectin-3. To test the relationship between galectin-3 and known AGE-binding proteins, we conducted Western blot analysis of purified recombinant murine galectin-3 with polyclonal antibodies raised against the purified 90-kD AGE-BP protein from rat liver membrane. A monoclonal antibody to galectin-3 (mAb M3/38), and likewise the polyclonal chicken anti-p90, but not an isotypic rat IgG2a, nor a normal chicken IgG, identified a 32-kD band in Western blots (Fig. 1). This was consistent with the possibility that p90 contains an epitope shared by galectin-3 or that the 90-kD protein consists of more than one polypeptide, one of which is galectin-3.

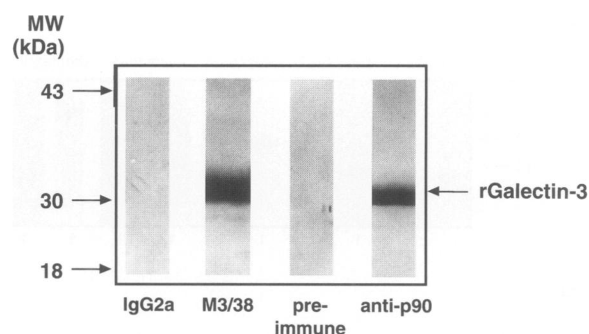

FIG. 1. Recombinant galectin-3 is recognized by anti-p90 antibody

Purified recombinant murine galectin-3 (1 μg/lane) was subjected to SDS-PAGE through a 12% gel and electroblotted onto nitrocellulose. After blocking with 2% BSA in PBS for 2 hr, the blots were exposed to each of the primary antibodies as shown: rat IgG2a; anti-galectin-3 mAb M3/38; purified avian serum IgG; or purified IgG fraction of avian anti-p90. Immunoreactivity was visualized by the appropriate alkaline phosphatase-conjugated goat anti-chicken IgG or mouse anti-rat IgG and NBT/BCIP color reaction.

To confirm and expand the question of whether galectin-3 or any other members of the galectin family, comprising of galectin-1 through -4 (19–21), exhibit AGE-binding activity, we performed ligand blot analysis of recombinant human galectin-1, -2, and -3, and rat galectin-4 using ^{125}I-AGE-BSA as ligand. As shown in Fig. 2A, galectin-3 and -4 (domain I), but not galectin-1 or -2, could bind AGE-BSA under identical conditions. Phosphorimage analysis (Fig. 2B) indicated that, among all, galectin-3 exhibits the strongest activity, while galectin-4

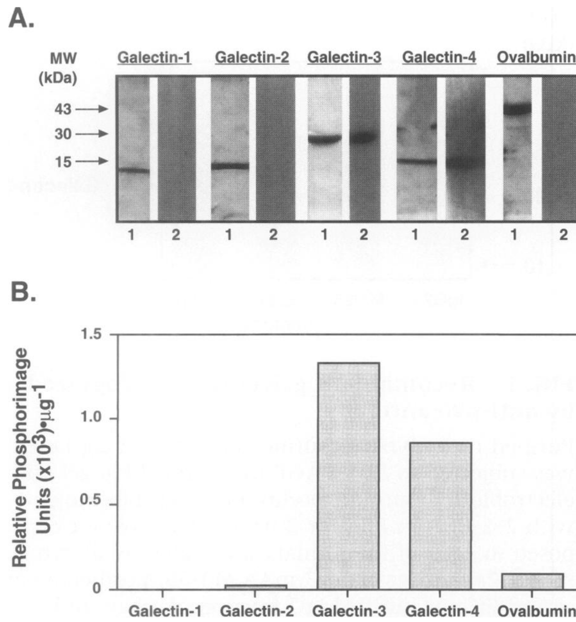

FIG. 2. Ligand blot analysis of galectins 1–4

Recombinant human galectin-1, -2, -3 and rat galectin-4 (domain I) were subjected to SDS-PAGE (4–20% gel, 5 μg/lane) and electroblotted onto nitrocellulose membrane. (A) Blots were either stained with Amido black or probed with ^{125}I-AGE-BSA. Protein staining of filters (1) and autoradiographs of ligand blots (2) are shown. (B) Comparison of ^{125}I-AGE-BSA binding to galectins by phosphorimage (PI) analysis. Data are expressed in relative PI units/μg protein.

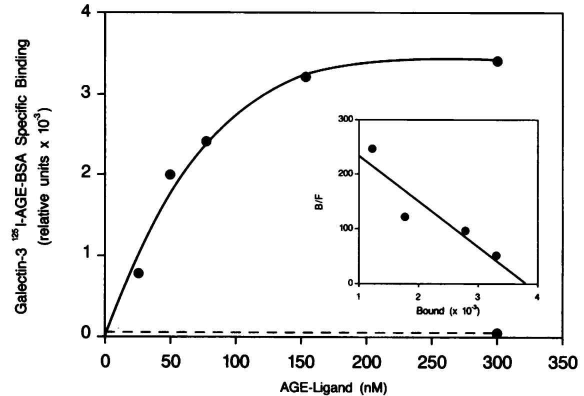

FIG. 3. AGE binding activity of galectin-3

Purified recombinant rat galectin-3 (3 μg/dot) was immobilized onto a nitrocellulose filter. After blocking with 2% BSA in blocking buffer for 2 hr filters were probed with increasing concentrations of ^{125}I-AGE-BSA (solid line) or ^{125}I-BSA (dotted line) in the presence or absence of 100-fold excess unlabeled ligand. After 1 hr, the filters were washed and counted for bound radioactivity. Specific binding was calculated by subtracting nonspecific from total filter-associated radioactivity. (Inset) Scatchard analysis of binding data (representative of three independent experiments).

recognized approximately 50% of the labeled AGE-ligand bound by galectin-3.

Recombinant Galectin-3 Binds AGE-Modified Proteins

To test whether galectin-3 itself exhibits AGE-specific binding activity, purified rat galectin-3 was immobilized onto nitrocellulose membrane (3 μg/dot) and probed with increasing amounts of ^{125}I-AGE-BSA or ^{125}I-BSA in the presence or absence of 50-fold excess unlabeled AGE-BSA or BSA native. As shown in Fig. 3, galectin-3 binding of ^{125}I-AGE-BSA rose to saturation in a concentration-dependent manner. Radioligand binding was completely abrogated by unlabeled AGE-BSA added in excess of 50-fold. Scatchard plot analysis of the binding data (Fig. 3, inset) were consistent with a single class of binding sites and an affinity of 3.5×10^7 M^{-1}. This affinity is of the same order of magnitude reported previously for the AGE-receptor on macrophages/monocytes (5).

Similar AGE-binding activity was exhibited by galectin-3 immuno-precipitated from RAW 264.7 cell extracts using mAb M3/38. The immuno-precipitated material was subjected to SDS-PAGE and, after transfer to nitrocellulose, to ligand blot and Western analysis. Under these conditions, four proteins with apparent molecular weights of 28, 32, 40, and 50 kD were precipitated by mAb M3/38, which displayed ^{125}I-AGE-binding activity on ligand blotting (Fig. 4A, Lane 2), and which are likely to represent various galectin-3 mono-, or multimeric forms (18), or galectin-3 complexed with other membrane proteins. Two of these (shown by arrows) exhibited crossreactivity with a polyclonal anti-CBP-35 antibody. By comparison, isotypic IgG2a antibody failed to precipitate any AGE-binding species (Fig. 4A, Lane 1).

To determine the domain responsible for AGE binding, recombinant galectin-3 was digested with collagenase (32,33) and the resulting 18-kD C terminus was used for ligand blot analysis. As shown in Fig. 4B, this portion of the molecule bound ^{125}I-AGE (Lane 1) in a manner that was inhibited in the presence of

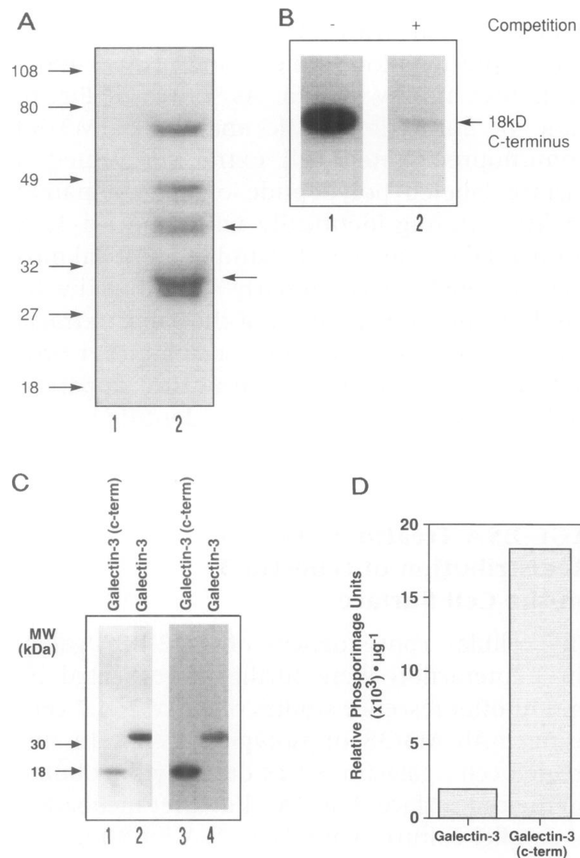

FIG. 4. Panel (A) NP-40 detergent extracts prepared from RAW 264.7 cells (2 × 10^5 cell equivalents/lane) were subjected to immunoprecipitation with either isotype control rat IgG2a (Lane 1) or anti-galectin-3 mAb M3/38 (Lane 2). After SDS-PAGE through a 12% gel and electrotransfer onto nitrocellulose, filters were subjected to ligand blotting using ^{125}I-AGE-BSA. (B) Purified 18-kD carboxyl-terminal domain of recombinant murine galectin-3 (3 µg/lane) was subjected to ligand blot analysis using ^{125}I-AGE-BSA in the presence (Lane 2), or absence (Lane 1) of 50-fold unlabeled AGE-BSA. (C) Filters were either stained with Amido black or probed with ^{125}I-AGE-BSA. Protein stain of the filters (1,2) and autoradiography of the ligand blots (3,4) are shown. (D) Quantitative analysis of ^{125}I-AGE-BSA binding to either intact galectin-3 or its 18-kD C-terminal peptide. Data from Panel C are expressed in phosphorimage units (PI)/mg protein.

50-fold excess unlabeled AGE-BSA (Lane 2), indicating AGE-binding specificity. In addition, when compared to the intact protein, the C-terminal fragment of galectin-3 exhibited stronger AGE-binding activity (Fig. 4 C and D); phosphorimage analysis indicated that the C terminus while retaining full AGE binding activity, as the intact protein, this is considerably higher than the binding activity of intact galectin-3. The finding suggests that the C terminus may contain the principal AGE-binding site domain of galectin-3 and the removal of collagen-like domain may facilitate the access of AGE-BSA ligand to this binding domain.

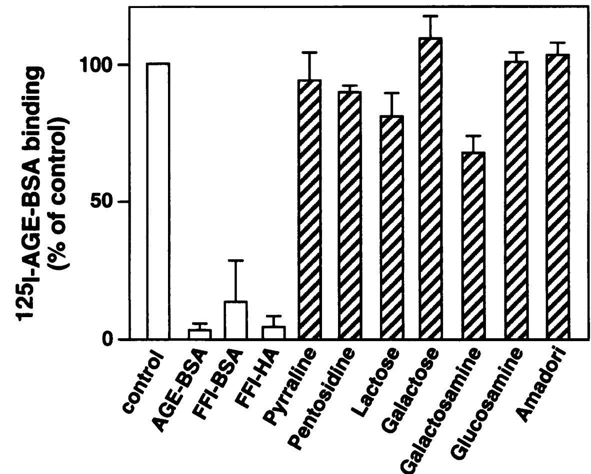

FIG. 5. Effect of carbohydrates on AGE-binding to galectin-3

Anti-Galectin-3 immunoprecipitated material from RAW 264.7 cells (2 × 10^5) was subjected to SDS-PAGE (12%) and ligand blot analysis using ^{125}I-AGE-BSA (300 nM) in the presence or absence or the indicated unlabelled competitors: AGE-BSA, FFI-BSA, FFI-HA at 50-fold excess (15 µM), lactose, galactose, galactosamine, and glucosamine at 100 mM, and Amadori product (1-deoxy-1-propyl-amino-fructose) at 1 mM. The amount of radioactivity associated with galectin-3 was determined by phosphorimage analysis of three identical experiments and expressed as percentage (mean ± SD) of ^{125}I-AGE-BSA bound in the absence of competitors.

Carbohydrates Are Not Effective Competitors of AGE-Binding to Galectin-3

Galectin-3 has been identified as a lectin with binding specificity to carbohydrate moieties such as lactose, galactose and galactosamine (21,22). To test whether the galectin-3 AGE-binding domain functionally overlaps with that for simple carbohydrates, competitive ligand blot inhibition experiments were performed. Equal amounts of galectin-3 immunoprecipitated from RAW cell detergent extracts were subjected to SDS-PAGE and electrotransferred onto nitrocellulose. ^{125}I-AGE-BSA ligand blot analysis was performed in the presence or absence of a large excess of unlabeled competitors. Compared with the complete inhibition achieved by unlabeled AGE-BSA

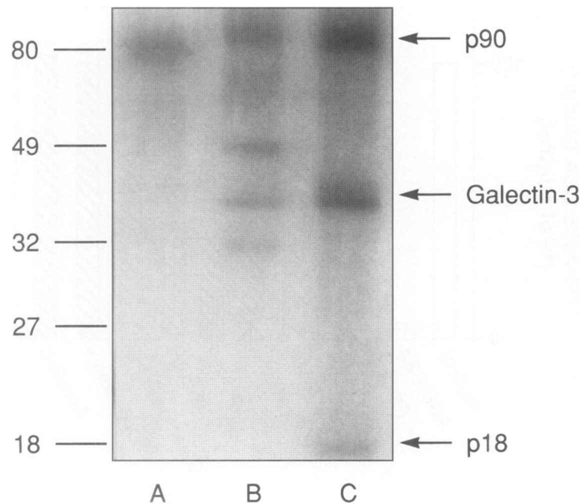

FIG. 6. Immuno- and ligand-affinity precipitation of surface radioiodinated RAW 264.7 cells
Detergent extracts prepared from surface radioiodinated RAW 264.7 cells were subjected to immunoprecipitation using isotype control rat Ig2a (Lane A), anti-galectin-3 mAb M3/38 (Lane B), or ligand-affinity precipitation using AGE-BSA-sepharose (Lane C). The precipitates were boiled in sample buffer containing 2.5% β-mercaptoethanol and electrophoresed through a 12% SDS-PAGE gel. Proteins were visualized by autoradiography.

and FFI-HA, a synthetic AGE recognized by macrophage AGE receptors, added either free or attached to BSA (FFI-BSA) (6,7) at 50-fold excess (15 μM), the addition of lactose, galactosamine, and glucosamine at more than 3×10^5-fold excess (100 mM) failed to significantly alter ^{125}I-AGE-BSA binding to galectin-3 (Fig. 5). Neither pentosidine (35) nor pyrraline (36), two other known AGEs competed for binding to galectin-3 (Fig. 5). Furthermore, a model synthetic product of the early steps of nonenzymatic glycation, 1-deoxy-1-propyl-amino-fructose (Amadori), also failed to compete with AGE-BSA binding at concentrations as high as 1 M, ruling out the possibility that similar early glycation structures present in AGE-proteins prepared in vitro contribute significantly to AGE binding by galectin-3 (Fig. 5).

Cell-Surface Galectin-3 Binds AGE-BSA

Galectin-3 has been variously described as a cytoplasmic, nuclear, surface-associated, and a secreted protein (18–22,26). To demonstrate that galectin-3, when present on the cell surface is capable of binding AGE-ligands, surface-iodinated RAW 264.7 cell extracts were subjected to immunoprecipitation with M3/38 mAb, or ligand-affinity precipitation studies using an AGE-BSA-Sepharose affinity system. As shown in Fig. 6, Lane B, autoradiographic analysis of M3/38 immunoprecipitated cell extracts revealed a surface labeled polypeptide of approximately 35 kD, running identically with galectin-3. A surface-label polypeptide band of identical molecular weight was similarly identified by ligand-affinity precipitation of the same extracts (Fig. 6, Lane C). In addition, using either procedure, a polypeptide with the apparent molecular weight of 90 kD was identified (p90) (Fig. 6B and C).

AGE-BSA Treatment Causes Redistribution of Galectin-3 on the Cell Surface

The cellular consequences of AGE-BSA/galectin-3 interaction were further investigated by immunofluorescence studies of RAW 264.7 cells using mAb M3/38 or isotypic control. In untreated cells, galectin-3 was diffusely distributed on the cell surface (Fig. 7A), but after incubation of parallel cultures with AGE-BSA for 30 min, a distinct pattern of patchy distribution of galectin-3 immunoreactivity was observed (Fig. 7B). In contrast, staining with the isotypic control was negative (Fig. 7C).

Interaction of AGE-Ligand with Cell Surface Proteins Results in Formation of High-Molecular Weight Aggregates

Galectin-3 has been shown to exhibit positive cooperativity in binding to multivalent glycoproteins, suggesting that the lectin has a tendency to self-associate resulting in dimers or oligomers (32,33). To inquire whether AGE-binding promotes similar aggregation of galectin-3, cellular membranes prepared from RAW 264.7 cells were incubated with ^{125}I-AGE-BSA or ^{125}I-FFI-BSA and at various intervals the mixture was subjected to SDS-PAGE through 12% gels under reducing conditions (2.5% β-mercaptoethanol). The formation of SDS-insensitive, β-mercaptoethanol-resistant aggregates was quantitated by phosphorimage analysis of the high-molecular weight material forming on the top of the gel. In contrast to incubations with unmodified BSA, which did not induce aggregate formation (Fig. 8, Lanes g and h), the interaction of ^{125}I-AGE-BSA or ^{125}I-FFI-BSA with membrane proteins

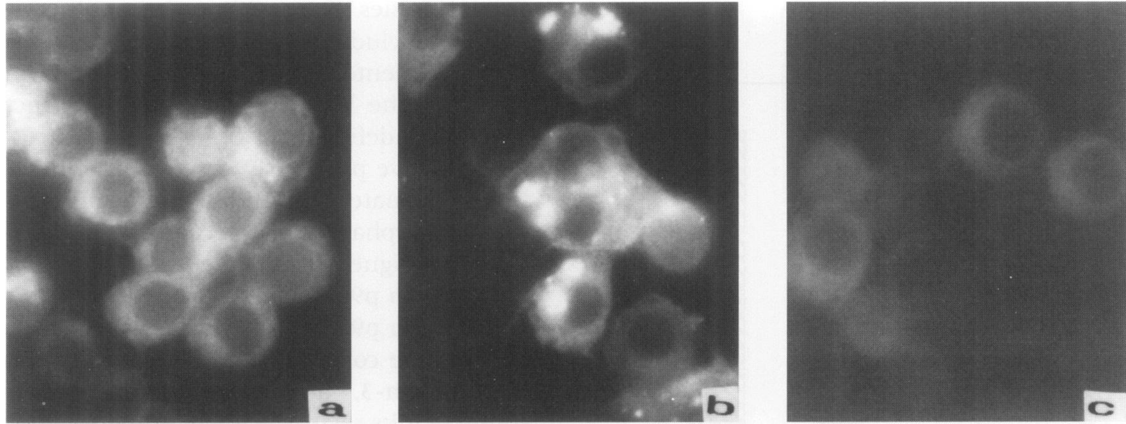

FIG. 7. Distribution of galectin-3 on RAW 264.7 cell surface by immunofluorence
Cells were treated with BSA (a) or AGE-BSA (b and c) for 30 min and probed with either anti-galectin-3 mAb M3/38 (a and b) or isotype control (c) followed by FITC-conjugated goat anti-rat IgG.

induced the formation of very high molecular weight complexes (Fig. 8, Lanes b–d). The addition of hydroxylamine, a known inhibitor of thioester linkage, at 100 mM prior to the addition of ^{125}I-FFI-BSA abrogated aggregate formation (Fig. 8, Lane f).

To investigate whether these complexes contained galectin-3, membrane-associated galectin-3 was also immunoprecipitated from RAW 264.7 cells by mAb M3/38, and radioiodinated before incubation with unlabeled AGE- or FFI-BSA. The interaction of this radiolabeled galectin-3 with either ligand resulted in the formation of SDS-, and β-mercaptoethanol-insoluble high-molecular weight complexes (Fig. 9 c and e). The addition of hydroxylamine (50 mM) prevented this aggregate formation (Fig. 9 d and f). Similarly, the interaction of recombinant galectin-3 with AGE- and FFI-BSA also led to the formation of large hydroxylamine-inhibitable aggregates (data not shown). Other nucleophiles, such as hydrazine (100 mM), 2-PAM (50 mM), and iodoacetamide (1 mM), also inhibited the formation of aggregates (data not shown).

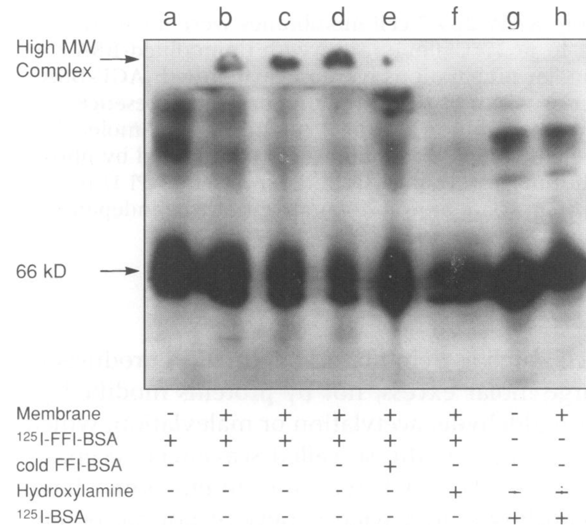

FIG. 8. AGE ligand induces membrane protein complex formation
RAW 264.7 cell membrane preparations were incubated with ^{125}I-FFI-BSA at 37°C for the indicated time. After adding SDS-PAGE buffer containing 5% β-mercaptoethanol, the samples were boiled and electrophoresed through a 12% gel. The proteins were visualized by autoradiography. ^{125}I-FFI-BSA was incubated without cell membrane (Lane a), with cell membrane for 1, 3, and 6 hr, respectively (Lanes b–d), in the presence of 50-fold unlabeled FFI-BSA for 6 hr (Lane e), in the presence of membrane extracts and hydroxylamine (100 mM, 6 hr) (Lane f). Unmodified ^{125}I-BSA alone (Lane g) or with cell membrane used as controls (6 hr) (Lane h). Very high molecular weight complex formation is indicated on top of gel by arrow. Data are representative of six independent experiments.

DISCUSSION

Initially, monocyte/macrophages and subsequently other cell systems were found to bind AGE-modified substrates with high-affinity saturable kinetics, implying the existence of specific receptor(s) (6–9). This binding is not antagonized by the presence of simple sugars, glycoconjugates or early glycation reaction prod-

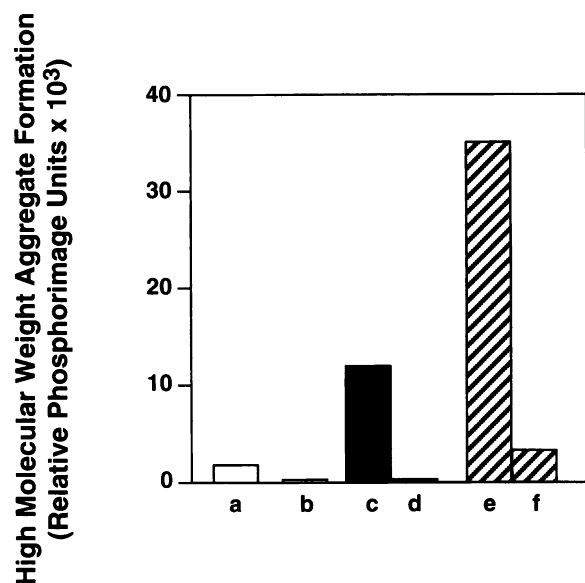

FIG. 9. Phosphorimage analysis of high-molecular weight aggregates forming during galectin-3 and AGE-ligand interaction

After radioiodination, galectin-3 immunoprecipitated from RAW 264.7 cell membranes were incubated for 24 hr at 37°C alone (a) or with unmodified-BSA (b). Similar preparations were incubated with AGE-BSA (c) or with FFI-BSA (e) alone or in the presence of hydroxylamine (100 mM) (d and f). High-molecular weight complex formation was quantitated by phosphorimage (PI) analysis and expressed as PI U/mg protein. Data are representative of three independent experiments.

ucts, such as Schiff bases or Amadori products in large molar excess, nor by proteins modified by formaldehyde, acetylation or maleylation, which define in part the so-called scavenger receptors (6–9,13,14). AGE-receptor interactions have been linked to a wide variety of normal potentially pathogenic responses, including migration, activation, cytokine and growth factor induction, and matrix protein secretion (4,5,8,9,14,15). These findings have further encouraged the identification and isolation of AGE-binding proteins and the molecular definition of the AGE-receptor complex (11–13,16,17).

Derived from the covalent attachment of reducing sugars, the advanced glycation products that form spontaneously on proteins or lipid substrates in vivo represent a structurally heterogeneous class of reactive adducts that can form infra- and intermolecular crosslinks (1–5). One such chemically defined AGE structure is the furoyl-furanyl imidazole (FFI), the first AGE-crosslink identified in vitro (27) as well as in animal tissues (28), while others subsequently isolated include the pyrrole containing AFGP (34), the pentosidine (35), and pyrraline (36); however, the large majority of AGE structures remains undefined.

We have previously described two polypeptides, designated p60 and p90, as components of the macrophage AGE-receptor (13). During studies designed to provide cDNA clones corresponding to p90 using antibodies made to purified rat liver p90, we repeatedly isolated a partial cDNA clone corresponding to galectin-3.

Galectin-3, a cellular protein previously studied for its relatively low binding activity for a broad range of simple carbohydrates (K_d 4 $\times$ 10^5 M^{-1}) (37) is found in the cell nucleus, the cytosol, on the cell surface, as well as extracellularly and has been implicated in events such as macrophage migration, adhesion, immune response modulation, growth and differentiation and is included to the Galectins, a family of lectins, sharing affinity for β-galactoside sugars (18–26). There has been no clear evidence however that galectin-3 acts as a surface receptor, since protein and cDNA sequence analysis of this molecule indicated neither transmembrane anchor sequences, nor signal peptide (38). Western blot analysis of recombinant murine galectin-3, using the same antisera developed against the rat liver 90-kD AGE-binding polypeptide (anti-p90) (13) that were used in expression screening revealed strong immunoreactivity between these antisera and galectin-3. This raised the possibility of either an immunologic cross-reactivity between p90 and galectin-3 or of immunoreactivity directed to galectin-3-like epitopes copurified with the p90 polypeptide.

The physical association of galectin-3 with AGE-receptor components versus an independent role of galectin-3 as an AGE-binding protein was investigated further. First, it was ascertained that galectin-3 possessed distinct binding affinity for AGE-modified albumin, a property which was not shared by two other members of the galectin family (galectin-1 and -2) and only partially by galectin-4. Then, evidence was obtained to show that purified recombinant galectin-3 is capable of independently and specifically binding AGE-modified proteins with saturable kinetics (K_d = 3.5 $\times$ 10^7 M^{-1}). This binding affinity was lower than the affinity observed in intact membrane preparations ($\times 10^{-9}$) (13), which may reflect either the need for a membrane localization or for cooperation of more than one of the AGE receptor components. Nevertheless, the affinity

of galectin-3 for AGEs is distinctly higher than that for carbohydrates ($K_d = 4 \times 10^5 \ M^{-1}$) (37), even when FFI-HA is used for comparison with monovalent sugars. In addition, based on competitive studies with previously known ligands of galectin-3 such as lactose, galactose, and other glycoconjugates added in large excess, the recognition site for AGE-moieties appeared to be distinct from those for carbohydrate moieties. At least one AGE-binding domain colocalizes to the same 18-kD C-terminal fragment of galectin-3 that contains the carbohydrate recognition domain of the molecule. Although our data do not distinguish between a single or multiple binding sites for AGE-modified ligands on galectin-3, the specificity of this protein for AGE-moieties was further defined in experiments using chemically defined synthetic "early" or Amadori, and "late" glycation intermediates (1–4) in competition experiments. Consistent with reports on lack of recognition by other AGE-binding proteins (6,8,13,14), Amadori failed here to displace AGE-ligands, thus eliminating the role of early glycation products in this interaction. In contrast, the model synthetic AGE ligands, FFI-HA and FFI-BSA (25,26), previously shown to be readily recognized by macrophages (6,7,11,12) competed very effectively. Surface binding of FFI-BSA has been associated with macrophage/monocyte activation, cytokine release (39) and autocrine AGE-receptor up-regulation (40), mimicking and even surpassing the identical activities of naturally formed AGE-BSA. Owing its efficiency most probably to the higher concentration of AGE-modifications per polypeptide compared to the naturally formed AGE-BSA, the validity of the synthetic FFI-BSA was confirmed while it also proved a valuable probe for the present studies.

AGE-affinity precipitation of surface-labeled cell extracts yielded polypeptides with identical mobility to galectin-3 at 35 and 90 kD, while immunoprecipitation with anti-galectin-3 revealed in addition, a 50-kD protein, which indicated the proximity, and possible association of galectin-3 with other AGE-binding polypeptides on the cell surface (e.g., p90 and p60). Alternatively, exposure of cells to AGEs may enhance the surface expression of galectin-3, an antigen, first recognized as a macrophage activation marker (21). Given the known property of AGEs to induce macrophage activation (39,40), this would not be inconsistent with this hypothesis. The latter was supported by immunofluorescence studies showing that upon exposure of RAW cells to AGE-BSA, the presence of galectin-3 on the cell surface intensified, while it mobilized to form discrete complexes. Based on the previously reported propensity of galectin-3 to associate with itself (18,32,37), we sought to evaluate whether AGE ligands may also promote galectin-3 complex formation.

Our results indicate that interaction of AGE- and FFI-modified BSA with either membrane-derived or recombinant galectin-3 resulted in the formation of high-molecular weight aggregates in a time-dependent manner. These complexes proved resistant to SDS or β-mercaptoethanol treatment but were completely inhibitable in the presence of nucleophiles, such as hydroxylamine, hydrazine, and 2-PAM. The lability of the complexes to hydroxylamine at an alkaline pH suggested that the interactions within the complexes may involve the formation of covalent bond between galectin-3 and other molecules, and that the bond is likely a thioester. The presence of such thioester linkages has been previously reported for complement factor C3 (41,42,43) and ubiquitin-activating enzyme E1 (44). Internal thioester bonds mediating the fixation of complement factor C3 to biological targets seem necessary for complement function (42,43). Similarly, the attachment of ubiquitin E1 and subsequent trafficking of target proteins to degradation pathways is also dependent on high energy thioester linkages (44). Although further characterization of the nature and the functional significance of the linkages formed between galectin-3 and AGE-modified proteins is necessary, it is possible to speculate that the formation of high energy thioester bonds may contribute to the efficient attachment, and uptake of proteins modified in vivo by a wide range of AGE moieties with variable affinities, to be subsequently escorted toward intracellular degradative compartments.

The presence of galectin-3 on the cell surface is interesting since there is no consensus transmembrane spanning domain present in its sequence (18,38). Thus, it is conceivable that this polypeptide is expressed on the cell surface in association with other subunits of the AGE-receptor complex which may contain such domains. In this regard, our immuno-precipitation and affinity studies using anti-galectin-3 and ligand blot analysis, showed that in addition to the p90 and to anti-galectin-3-reactive polypeptides, two other species with approximate molecular weights of 40 and 50 kD were associated with galectin-3 and were capable of binding AGE-BSA.

The identity of these molecules is under investigation; however, they are likely to correspond to one or more of the already characterized macrophage surface AGE-binding proteins (13,16). Thus far, the primary role of galectin-3 has remained unclear, although it has been suggested that galectin, among others, may play a role in macrophage adhesion to basement membranes and subsequent migration toward the site of inflammation via interaction with laminin (45), or that it may be associated with neoplastic progression of colon carcinoma (46).

Collectively, our data provides evidence of a novel class of in vivo forming ligands for galectin-3, advanced glycation end products. The physiologic significance of this interaction is under further investigation. However, the observation that galectin-3 exhibits high-affinity binding activity for AGE-modified proteins, constitutes a novel activity for this molecule and introduces it as a new member of the macrophage receptor system for AGE-modified senescent macromolecules.

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