The Effect of Nonenzymatic Glucosylation on the Binding of the Main Noncollagenous NC1 Domain to Type IV Collagen*

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Type IV collagen has the ability to self-assemble by amino end, carboxyl end, and lateral associations to complex network-like structures which can be visualized by rotary shadowing. The main noncollagenous NC1 domain which is located at the carboxyl end of type IV collagen molecules binds to itself to form dimers and also binds along the length of type IV collagen. The latter binding initiates lateral assembly. Following in vitro nonenzymatic glucosylation of the isolated NC1 domain, binding to the helix-rich domain of type IV collagen was impaired. In turbidity experiments, the nonenzymatically glucosylated NC1 domain minimally suppressed the development of turbidity of collagen solutions when compared to control NC1 domain. In rotary shadowing experiments nonenzymatically glucosylated NC1 domain did not significantly inhibit lateral associations or networks formed by type IV collagen, whereas control NC1 domain caused a drastic decrease in laterally assembled structures. These data suggest that nonenzymatic glucosylation of the NC1 domain may interfere with normal assembly of type IV collagen in diabetes mellitus and may be related to abnormal functions of basement membranes in this pathological condition.

Nonenzymatic glucosylation is characterized by the non-enzymatic binding of glucose primarily to the ε-amino groups of lysine residues in long-lived proteins (1). This process is accelerated in diabetes mellitus (1, 2). Nonenzymatic glucosylation has been observed in basement membranes, i.e. the glomerular basement membrane (GBM) of the kidney, in diabetes (3). This particular basement membrane forms the only barrier to the extravasation of plasma proteins into the urine (4-6), and during later stages of diabetes, it often becomes permeable to albumin and other larger plasma proteins leading to proteinuria. The reasons for this impairment of permselectivity are not understood mainly because the molecular basis of this function remains largely unknown. However, it is known that basement membranes are assembled from distinct macromolecules, several of which have the ability to self-assemble and also bind to each other (7). Type IV collagen, a large glycoprotein of basement membranes (M, 500,000), is believed to form the main supportive framework of these structures (6). It has the ability to polymerize by end-to-end (8) and lateral associations (9, 10) and forms a complex network upon which other components of basement membrane (such as laminin, entactin/nidogen, and heparan sulfate proteoglycan) can be assembled. Nonenzymatic glucosylation has been shown to occur in type IV collagen extracted from the GBM (11-14). Type IV collagen has a major pepsin-resistant discontinuous triple helical domain (9, 15) and another major noncollagenous NC1 collagenase-resistant domain (8). The triple helical domain serves for associations between the amino ends of four adjacent collagen molecules ("7s" tetrameric spiders (8, 9)) and for lateral associations between adjacent type IV collagen molecules (7, 9). The NC1 domain functions to bind to the carboxyl end of an adjacent collagen molecule (8) and also to bind along the length of the triple helical part (10). As a result of these associations, a complex irregular network forms, which can be visualized by rotary shadowing (9, 10).

The purpose of this study was to examine the effects of in vitro nonenzymatic glucosylation of the NC1 domain on the process of polymerization of type IV collagen. The hypothesis was tested that this chemical modification leads to altered ultrastructural assembly of this protein into a network.

MATERIALS AND METHODS

Isolation of Type IV Collagen—Type IV collagen was purified from the lathyritic Engelbreth-Holm-Swarm tumor according to the modification of Kleinman et al. as previously described (8, 10, 16). Animals were rendered lathyritic by the addition of 0.25% β-aminopropionitrile fumarate (Sigma) in their drinking water. 250-350 g of collected Engelbreth-Holm-Swarm tumor was homogenized in a solution containing 50 mM Tris-HCl, pH 7.4, 3.4 mM NaCl, 50 μg/ml p-phenylmethylthanesulfonyl fluoride, 50 μg/ml chloromercuribenzoate, 1 mM EDTA. The homogenate was centrifuged at 10,000 rpm for 30 min, and the pellet was collected and washed with the same buffer 5 times. The final pellet was extracted with 2 mM guanidine HCl (Sigma) in 50 mM Tris-HCl, pH 7.4, containing 50 μg/ml phenylmethylthanesulfonyl fluoride, 50 μg/ml chloromercuribenzoate, 1 mM EDTA, and subsequently with 2 mM guanidine HCl, 2 mM dithiothreitol in 50 mM Tris-HCl, pH 7.4, containing the same mixture of protease inhibitors. The final extract was incubated with DEAE-52 (Whatman), in 4 mM urea, 0.25 mM NaCl, 50 mM Tris, pH 8.6, containing protease inhibitors as mentioned above for 10 h at 4 °C with gentle rocking. The suspension was centrifuged, and the supernatant was collected and dialyzed against 0.05 M Tris-HCl, pH 7.4, containing 2 mM guanidine HCl, 2 mM dithiothreitol, 1 mM EDTA, 50 μg/ml phenylmethylthanesulfonyl fluoride. Following diafiltration, type IV collagen was centrifuged at 40,000 rpm in a Beckman L2-65B ultracentrifuge rotor (Ti 40) for 90 min to remove large aggregates (>50 S), and the supernatant was stored on ice in the same buffer until further use. Protein concentrations were determined by the method of Lowry et al. (17).
were determined by amino acid analysis and by the method of Waddell (17).

Isolation of the NC1 Domain of Type IV Collagen—The main noncollagenous domain (NC1) was isolated from intact Engelbreth-Holm-Swarm-derived type IV collagen by the use of bacterial collagenase (CLSPLA, Cooper Biomedical) according to published procedures (10, 12). Type IV collagen was dialyzed against 100 ml of HCl at a rate of 1.5 ml/min) (23). Amino acids were resolved with an acetonitrile gradient from 9 to 85% acetonitrile in 50 mM NaH2PO4, pH 7.4, containing 0.2 M NaCl and 2 mM CaCl2 and was incubated with collagenase (substrate/enzyme ratio, 50:1) at 37 °C for 48–70 h with gentle rocking. The solution was then centrifuged at 10,000 rpm for 30 min to remove aggregated material, and the supernatant was concentrated with Aquacide IIA (B. D. Biosciences). Lyophilized glucosylated amino acids were derivatized for 1 h with 0.025 M NaOH according to published procedures (21). The derivatized NC1 domain, the perimeter of each population of NC1 particles was measured from photomicrographs at a final magnification of 600,000 using an Optomax semiautomatic image analysis system (Optomax) equipped with an Apple IIe computer. Histograms of each population were plotted. Statistical evaluation of each class of particles was done by the Student’s t test (24).

Control NC1 domain was incubated in PBS containing 10 mM EDTA in the presence of 10 or 100 mM glucose for 10–25 days at 38 °C. In several instances, 1 ml of d-[-1H]glucose (Amersham Corp.) was added in the incubation buffer. Before use d-[-1H]glucose was incubated for 5 days with 1 mg/ml BSA (Sigma) in PBS to remove possible contaminants (20). Free d-[-1H]glucose was separated from BSA by gel filtration through a Sephadex G-25–50 (Sigma) column (1 × 20 cm) equilibrated in PBS. In all instances, 5–8% of the isotope was incorporated into BSA under these conditions. This procedure has been reported to remove possible radiolabeled contaminants of unknown nature which may be present in solutions of d-[-1H]glucose (20). d-[-1H]glucose was incubated with NC1 domain immediately after purification from contaminants.

In Vitro Nonenzymatic Glucosylation—The NC1 domain (=500 µg/ml) was incubated in PBS containing 10 mM EDTA in the presence of 10 or 100 mM glucose for 10–25 days at 38 °C. In several instances, 1 ml of d-[-1H]glucose (Amersham Corp.) was added in the incubation buffer. Before use d-[-1H]glucose was incubated for 5 days with 1 mg/ml BSA (Sigma) in PBS to remove possible contaminants (20). Free d-[-1H]glucose was separated from BSA by gel filtration through a Sephadex G-25–50 (Sigma) column (1 × 20 cm) equilibrated in PBS. In all instances, 5–8% of the isotope was incorporated into BSA under these conditions. This procedure has been reported to remove possible radiolabeled contaminants of unknown nature which may be present in solutions of d-[-1H]glucose (20). d-[-1H]glucose was incubated with NC1 domain immediately after purification from contaminants.

Control NC1 domain was incubated in PBS containing 10 mM EDTA, in the absence of glucose, under similar conditions. After the end of the incubation period, all solutions were dialyzed exhaustively against PBS at 4 °C, and the protein was stored at −70 °C until further use.

Tracking of d-[-1H]Glucose Incorporation in Lysine Residues by Glucosylated Lysine Standards—Poly-L-lysine hydrobromide (Mw = 40,000, Sigma) was incubated at 5 mg/ml in PBS containing 10 mM EDTA in the presence of 100 mM glucose and 1 mM of d-[-1H]glucose at 28 °C for 30 days. Excess glucose was removed by exhaustive dialysis against PBS at 4 °C. Glucosylated poly-L-lysine as well as the glucosylated NC1 domain were subsequently dialyzed against 0.1 M sodium phosphate buffer, pH 7.0. They were then incubated in the presence of 200 nM excess sodium borohydride (Sigma) dissolved in 0.2 M NaOH according to published procedures (21). The incubation time was 1 h (10 min at room temperature, 50 min on ice). At the end of the incubation, all solutions were dialyzed exhaustively against 0.1 M phosphate buffer, pH 7.0, followed by dialysis against 0.1 M ammonium bicarbonate (pH 7.4) and then lyophilized. Lyophilized samples of the NC1 domain or poly-L-lysine were hydrolyzed with 6 N HCl in evacuated tubes for 24 h at 110 °C, and HCl was evaporated with nitrogen. The hydrolysate was reconstituted in 1.5 ml of 0.025 M sodium phosphate buffer adjusted to pH 7.9 and chromatographed on a 0.5 × 5-cm boronate column (Affi-Gel 601, Bio-Rad) which selectively binds cis-diol groups of sugar residues that remain bound to amino acids (22). The column was washed for 8–10 h with 0.025 M sodium phosphate buffer at a flow rate of 30 ml/h, and bound glucosylated amino acids were then eluted with 100 ml of 0.025 M HCl according to published procedures (22, 23). Fractions of 0.5 ml each were collected and 20 µl of each fraction were counted in 5 ml of Ecolite (West Chem, San Diego, CA) using a Beckman LS-3801 liquid scintillation counter.

HPLC Amino Acid Analysis—Amino acid analysis was performed by o-phthalaldehyde (Pierce Chemical Co.) precolumn derivatization followed by separation on a C18 reverse-phase column (Beckman) (23). Lyophilized glucosylated amino acids were derivatized for 1 min with a solution containing 25 mg of o-phthalaldehyde in 2.5 ml of HPLC grade methanol (Burdick and Jackson), 250 µl of sodium borate, and 25 µl of ethanolethiol (Pierce Chemical Co.). Derivatized samples were then injected into a Beckman HPLC system for identification according to previously published procedures (22, 23). Amino acid mixture (8–10 µl of sample) was modelled with an amphoteric gel was run at a constant current of 50% versus an aqueous phase of 0.0125 M NaH2PO4, pH 7.2, at a flow rate of 1.5 ml/min) (23). Amino acid standards were processed identically.

Turbidity Measurements—Type IV collagen and the isolated NC1 domain, either control or glucosylated, were dialyzed against PBS at 4 °C overnight and were then centrifuged at 38,000 rpm in a Beckman L2-66B ultracentrifuge (Ti 40) for 30 min at 3 °C to remove large aggregates. Type IV collagen only or mixtures of type IV collagen and either control or glucosylated NC1 domain were incubated for various concentrations of assembled type IV collagen polymers (9). We have observed by varying the glycerol concentration that 20% glycerol has minimal effects on networks formed by type IV collagen and also allows for good visualization of these proteins. After the addition of all samples was taken to help preserve the network structures formed by incubated intact type IV collagen. This concentration was taken to be 10% because higher glycerol concentrations have been reported to have a disruptive effect on assembled type IV collagen polymers (9). We have observed by varying the glycerol concentration that 20% glycerol has minimal effects on networks formed by type IV collagen and also allows for good visualization of these proteins. After the addition of all samples was taken to help preserve the network structures formed by incubated intact type IV collagen. This concentration was taken to be 10% because higher glycerol concentrations have been reported to have a disruptive effect on assembled type IV collagen polymers (9).

To statistically evaluate the size of the both the control and glucosylated NC1 domain, the perimeter of each population of NC1 particles was measured from photomicrographs at a final magnification of × 500,000 using an Optomax semiautomatic image analysis system (Optomax) equipped with an Apple IIe computer. Histograms of each population were plotted. The mean of all fields was taken to be the number of fields containing laterally self-assembled or randomly aggregated type IV collagen in samples of type IV collagen alone and type IV collagen co-incubated with BSA, control, or glucosylated NC1 domain, each mesh of the grid was assumed to be divided into four equal sectors. If none or only a small field of one sector (less than 5%) was observed to contain laterally associated arrays of type IV collagen, the total field comprising all four sectors was classified as negative (0%). If one to three of the individual mesh sectors were observed to contain regularly assembled networks of type IV collagen the number of individual positive fields was counted, and the sector was classified as being partly positive accordingly (i.e. 25, 50, 75%, etc.). If all four parts of the mesh were found to contain extensive networks, the field was classified as positive (100%). At least 87 fields, each comprising a whole mesh divided into four equal sectors, were measured, and the final percentage measurements the mean of all measurements.

SDS-GElectrophoresis—Control and glucosylated NC1 domain were examined by SDS-polyacrylamide gel electrophoresis on 10% or 2–15% gradient gels according to the method of Laemmli (25). Protein samples were loaded on 200 µl wells, and the gel (containing also a 2% stacking gel) was run at 300–350 mA until the bands were clearly detected by fluorography after impregnating the gels with ENHANCE (Du Pont-New England Nuclear).
RESULTS

Isolated NC1 domain of type IV collagen was glucosylated \textit{in vitro} by incubation in the presence of 10 or 100 mM D-[6-\(^3\)H]glucose at 28 °C for various time intervals. Incorporation of D-[6-\(^3\)H]glucose in the NC1 domain incubated in the presence of 100 mM glucose was tested by SDS-polyacrylamide gel electrophoresis on 10% gels followed by fluorography (Fig. 1). D-[6-\(^3\)H]glucose was preincubated with BSA to absorb possible contaminants. Based on the specific activity of D-[6-\(^3\)H]glucose, which exist per dimeric NC1 domain.

3H]glucosylated NC1 domain, 4–6 lysine residues were modified per molecule of dimeric NC1 domain. This number corresponds to glucosylation of 10–12% of the lysine residues which exist per dimeric NC1 domain.

The hypothesis that glucose was incorporated at the \(\alpha\) amino groups of lysine residues, as reported elsewhere (12, 22, 23), was tested by HPLC analysis of \textit{in vitro} glucosylated poly-L-lysine. As published previously by many investigators (12, 22, 23), nonenzymatically glucosylated polylysine contains several glucosyl-lysine residues which can be inferred from the mobility of the products relative to the nonmodified polymer (12, 22, 23). When examined with this method, a prominent peak corresponding to glucosyl-lysine was present in glucosylated polypeptide controls. Glucosylated NC1 domain was found to contain a minor yet discrete peak corresponding to glucosyl-lysine (chromatographic data not shown). Based on the peak corresponding to glucosyl-lysine, 4.8 lysine residues/mol of NC1 domain were glucosylated.

When examined by turbidimetry, intact type IV collagen in PBS at 35 °C was observed to readily raise turbidity without a lag phase, as described previously (9, 10) (Fig. 2). Solutions containing both type IV collagen and control NC1 domain, which were incubated at 28 °C in the absence of glucose, developed drastically reduced maximal turbidity, as described in a previous report (10). For example, when control NC1 domain was co-incubated with type IV collagen at a molar ratio of 0.8, it decreased the development of turbidity of type IV collagen by approximately 45% (Fig. 2). In contrast, the presence of the NC1 domain which was previously incubated with either 10 or 100 mM glucose (28 °C, 15 days) in solutions of type IV collagen drastically reduced the ability to suppress the development of maximal turbidity of type IV collagen.

The relative inability of nonenzymatically glucosylated NC1 domain to decrease the maximal turbidity of intact type IV collagen was proportional to the concentration of glucose during the \textit{in vitro} nonenzymatic glucosylation of the NC1 domain. For example, NC1 domain which was incubated in the presence of 10 mM glucose (28 °C, 15 days) was observed to decrease turbidity of intact type IV collagen by approximately 21% when compared to a 45% decrease caused by control NC1 domain. The NC1 domain which was incubated in the presence of 100 mM glucose (28 °C, 15 days) caused only an 11% decrease of maximal turbidity of type IV collagen. In all these instances the molar ratio of control or glucosylated NC1 domain to type IV collagen was maintained at 0.8.

In rotary shadowed images, prewarmed intact type IV collagen by itself was observed to form networks in ?73% of the fields examined. When NC1 domain was co-incubated with intact type IV collagen, it was observed to bind along the length of type IV collagen molecules with the previously described 100-nm periodicity (5, Fig. 4A). When assembly of type IV collagen to networks was examined quantitatively in the presence of control NC1 domain, only ?29% of the fields were observed to contain lateral associations or networks of type IV collagen (Figs. 3 and 4B). These data indicate that binding of the isolated NC1 domain to type IV collagen competes for the binding of native type IV collagen molecules and blocks subsequent lateral assembly.

NC1 domain which was incubated in the presence of 100 mM glucose was observed to bind only minimally to type IV collagen (Fig. 4C). Network formation in the presence of 4304

\textbf{Fig. 1.} Left, SDS-polyacrylamide gel electrophoresis (10% gel) of control (lane 1) and nonenzymatically glucosylated (100 mM glucose, 1 mCi of D-[6-\(^3\)H]glucose, 28 °C, 23 days) NC1 domain (lane 2) under reducing conditions. Right, fluorogram of lane 2 of the previous gel. Note incorporation of glucose in monomeric and dimeric forms of NC1 domain as well as in the bands of larger size (cross-linked adducts). \textit{Horizontal bars} represent mass standards in kDa: they are phosphorylase \(b\) (94 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and trypsin inhibitor (21 kDa).

\textbf{Fig. 2.} Turbidity of intact type IV collagen incubated under control conditions and in the presence of control or glucosylated NC1 domain. Type IV collagen (250 \(\mu\)g/ml) was incubated at 35 °C for 2 h in PBS alone (●—●), in the presence of 60 \(\mu\)g/ml control NC1 (Δ—Δ), in the presence of 60 \(\mu\)g/ml NC1 incubated for 10 days with 10 mM glucose (○—○), and in the presence of 60 \(\mu\)g/ml NC1 incubated for 10 days with 100 mM glucose (▲—▲). The NC1 domain was preincubated with or without glucose in PBS containing 10 mM EDTA at 28 °C.
Glucosylated NC1 domain was observed in 67% of the fields examined (Figs. 3 and 4D). These data indicate that nonenzymatic glucosylation of NC1 domain resulted in impairment of its binding to intact type IV collagen.

Individual molecules of isolated control NC1 domain, when examined by the technique of rotary shadowing, had an average perimeter of -65 nm. A few particles (6%) had a larger perimeter under control conditions. However, following in vitro nonenzymatic glucosylation, a small but statistically significant population (approximately 12%) of NC1 particles had a larger perimeter (>80 nm, Figs. 5 and 6, a and b). This indicated the presence of aggregated material following incubation in the presence of glucose. To test whether this was due to the presence of covalent bonds induced by nonenzymatic glucosylation (2, 26), control and in vitro glucosylated NC1 domain were examined by SDS-polyacrylamide gel electrophoresis on 2–15% gradient gels. It was observed that under reducing conditions control NC1 domain did not contain forms higher than dimers (M, 50,000) (Fig. 7). However, in all instances nonenzymatically glucosylated NC1 domain showed the presence of several nonreducible adducts of higher molecular weight between 75,000 and 125,000.

**DISCUSSION**

The main noncollagenous NC1 domain of type IV collagen has been described to be important for self-assembly of this glycoprotein; this self-association occurs when two adjacent NC1 domains bind to each other and result in dimer formation (8) and when the NC1 domain binds along the length of type IV collagen molecules, which is required for lateral associa-

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**FIG. 3.** Diagrammatic representation of the percentage of fields containing lateral associations in various samples of prewarmed type IV collagen. From bottom to top: type IV collagen only, type IV collagen co-incubated with BSA, type IV collagen co-incubated with control NC1 domain, type IV collagen co-incubated with glucosylated NC1 domain. N represents the number of total fields examined.

**FIG. 4.** Rotary shadowing images of intact type IV collagen (150 μg/ml) incubated in PBS (35 °C, 1 h) in the presence of control NC1 (75 μg/ml) or NC1 incubated with 100 mM glucose (28 °C, 17 days, 75 μg/ml). A and B, control NC1 domain (incubated in PBS for 17 days at 28 °C) bound to type IV collagen with an apparent periodicity of 100 nm (A) and inhibited lateral association and network formation (B). C and D, glucosylated NC1 domain could not efficiently inhibit lateral association (C) and network formation (D) by type IV collagen. Bar equals 100 nm.
Nonenzymatic Glucosylation of NC1 Domain of Type IV Collagen

FIG. 5. Rotary shadowing images of control (A) and nonenzymatically glucosylated (B) NC1 particles. Isolated NC1 domain was incubated with 100 mM glucose (or without glucose in the case of control NC1) in PBS containing 10 mM EDTA at 28 °C for 15 days. In B arrows indicate larger NC1 globules. Bar equals 100 nm.

tions to occur (10). In this study, we addressed the question of whether nonenzymatic glucosylation of the NC1 domain impairs any of its known functions. It is known that in diabetes mellitus, particularly at later stages of the disease, the GBM loses its function of permselectivity, and proteinuria occurs (6, 27). Furthermore, in diabetes mellitus of long standing duration extensive thickening of the GBM occurs (6, 27). Whether these functional and structural changes are related to chemical or other modifications of basement membrane components remains to be elucidated. Nevertheless, it has been established that a chemical modification, that of excessive nonenzymatic glucosylation, occurs in whole GBM of diabetic rats (3) as well as in type IV collagen extracted from the GBM of diabetic rats (11, 12) and diabetic human subjects (13, 14). Large pepsin-extracted fragments of type IV collagen were observed to be nonenzymatically glucosylated as well (14). Type IV collagen extracted with this approach lacks the NC1 domain, which is pepsin-sensitive.

Because type IV collagen cannot be extracted intact from basement membranes due to extensive cross-linking of these structures, we used Engelbreth-Holm-Swarm-derived intact type IV collagen to study the process of self-assembly. This process has been examined in detail as previously reported (9, 10). We reported that binding of isolated NC1 domain to type IV collagen results in a dose-dependent decrease of the maximal turbidity of type IV collagen warmed at 35 °C in neutral buffers (10). In addition, we now present evidence that the isolated NC1 domain decreases network formation by type IV collagen. Quantitative analysis of rotary-shadowed images from samples of type IV collagen co-incubated in the presence of NC1 domain indicates that the binding of the NC1 domain to type IV collagen drastically decreases ultrastructural assembly to networks (Fig. 3). These data corroborate the hypothesis that the binding of isolated NC1 domain along the length of type IV collagen molecules competes for the binding of native type IV collagen and thus inhibits subsequent assembly to aggregates which form for the most part irregular polygonal structures (9). Incubation of isolated NC1 domain in the presence of varying amounts of glucose results in nonenzymatic incorporation of glucose; 4–6 lysine residues/
molecule of NC1 became modified following incubation in the presence of 100 mM glucose over a period of 15 days at 28 °C, and the presence of glucoseyl-lysine could be demonstrated. SDS-polyacrylamide gel electrophoresis of the NC1 domain incubated with glucose showed that the NC1 domain is intact (Figs. 1 and 7). In addition to chemical modification of the NC1 domain by nonenzymatic glucosylation, a dose-dependent impairment of the binding of NC1 domain to type IV collagen was observed. This was evident from the impaired ability of glucosylated NC1 domain to decrease maximal turbidity of type IV collagen following co-incubation of these two components at elevated temperatures (Fig. 2). Decreased turbidity of type IV collagen solutions is interpreted as reduction of self-association. Control NC1 domain, which was incubated under similar conditions but in the absence of glucose, maintained the ability to compete with the NC1 domain of native type IV collagen molecules and thus substantially decreased the development of maximal turbidity of this protein (Fig. 2).

In vitro nonenzymatically glucosylated NC1 domain bound only minimally to type IV collagen as determined by rotary shadowing and did not decrease network formation substantially (67% networks formed, compared to 71% networks present in prewarmed type IV collagen alone and ≈73% networks present in type IV collagen co-incubated with BSA), whereas control NC1 domain decreased network formation to a final percentage of ≈29% (Fig. 3).

Thus, both by rotary shadowing and turbidimetry, the binding of the NC1 domain to type IV collagen is severely impaired following in vitro nonenzymatic glucosylation.

An additional observation was the presence of small amounts of nonreducible cross-linked products in in vitro glucosylated NC1 domain as determined by SDS-gel electrophoresis and rotary shadowing (Figs. 5–7). It is well established that the process of nonenzymatic glucosylation leads to protein cross-linking which is mediated by chemically modified (glucosylated) lysines (2, 26). Thus long-lived proteins slowly incorporate glucose primarily at the ε-amino groups of their lysine residues. Eventually these modified lysine residues can bind an unmodified or another modified lysine residue covalently by either intra- or intermolecular bonds (2, 26, 28, 29). Both these changes resulting from lysine glucosylation and subsequent cross-linking are greatly enhanced in diabetes mellitus (2).

The fact that in vitro glucosylated NC1 domain undergoes glucose-induced cross-linking indicates that this process may be involved in impairing the binding of the NC1 domain to type IV collagen. However, the amount of observed cross-linked products was relatively small when compared to the degree of impairment of binding of this domain to type IV collagen. For example, only 12% of glucosylated NC1 particles had a significant increase of their perimeter compared to 6% of NC1 particles that had an increased perimeter under control conditions; the ability of NC1 domain glucosylated under similar conditions (100 mM glucose, 28 °C, 15 days) to suppress the development of turbidity of type IV collagen was diminished by 11% when compared to a 45% decrease of the development of turbidity by an equal concentration of control NC1 domain. Finally, glucosylated NC1 domain could not inhibit network formation beyond control values (approximately 70%, Fig. 3). Apparently it is difficult to quantitatively compare the results from the above mentioned three different approaches. It is obvious, however, that the degree of impairment of the binding of the NC1 domain to type IV collagen is not proportional to the observed amount of cross-linked products induced by nonenzymatic glucosylation. We speculate that cross-linked adducts may cause some masking, leading to unavailability of the binding site(s) in the NC1 domain under these conditions. However, as mentioned before our data can be explained only partially by this modification. It is possible that other factors are also involved in impairing the function of NC1 domain in binding to type IV collagen. For example, the possibility exists that a primary site of modification is a lysine residue which could be close to a site that mediates binding of the NC1 domain to the helix-rich domain of intact type IV collagen or could be a part in itself of the molecular determinant. Chemical modification of such a lysine residue would lead to substantially decreased binding, similar to what we observed both by turbidimetry and rotary shadowing. Alternatively, nonenzymatic glucosylation of a lysine residue might lead to conformational changes away from the site of modification which could involve the binding site.

Several studies are currently in progress to test this hypothesis. Other proteins have been reported to undergo either conformational changes or alterations of primary functional determinants following nonenzymatic glucosylation. For example, in vitro and in vivo diabetically modified albumin was shown to incorporate glucose nonenzymatically primarily at lysine 525 and to undergo a conformational change evident by fluorescence emission (30). Nonenzymatic glucosylation of albumin caused a decline in binding (a) to bilirubin and (b) to a long chain fatty acid, cis-parinaric acid. Furthermore, in vitro nonenzymatic glucosylation of the active peptide of fibrin resulted in reduced susceptibility to degradation by plasmin, apparently by direct modification of a lysine residue.
which is required for the binding of fibrin to plasmin (31).

In summary, under the experimental conditions described herein, in vitro nonenzymatic glucosylation of the NC1 domain affects one of its known functions, binding to the helix-rich part of type IV collagen. Since the native NC1 domain of intact type IV collagen was observed to mediate lateral assembly and network formation of this protein (10), it is tempting to speculate that the assembly of type IV collagen would be altered following in vitro and in vivo nonenzymatic glucosylation. Several questions remain to be answered to test the latter hypothesis. For example, is the binding site in the rodlike helix-rich part of type IV collagen also modified following in vitro glucosylation of type IV collagen? Or are there other sites, such as in the 7S amino-terminal area of type IV collagen, also modified by nonenzymatic glucosylation? These possibilities have to be considered since the intact helix-rich and 7S areas are also required for correct ultrastructural assembly of type IV collagen to higher ordered structures (8–10).

Finally, although it is known that under in vivo diabetic conditions nonenzymatic glucosylation of type IV collagen occurs (3, 11–14), does it occur in both the NC1 and the helix-rich domains? Also, are the in vivo modifications similar to those which we observed following in vitro nonenzymatic glucosylation? Does the process of nonenzymatic glucosylation randomly affect lysine residues or is there a particular lysine residue which is glucosylated? Although definitive information to answer the latter question is lacking, we have obtained preliminary evidence that at least one particular lysine residue from the α1-NC1 chain should be primarily glucosylated (32). These questions need to be answered to test the hypothesis that under diabetic conditions, assembly of type IV collagen which provides a supportive backbone of most basement membranes may be altered or impaired. Our data indicate that under conditions of in vitro nonenzymatic glucosylation, the ultrastructural assembly of type IV collagen to a complex network is impaired, perhaps by more than one mechanism. These changes could have a relationship to the abnormal function of basement membranes in diabetes mellitus.

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