In this study we demonstrate that the binding region of recombinant truncated human bone osteonectin (tHON) for type V collagen resides between amino acids 1 and 146. After removal of oligosaccharide chain structures from tHON, bovine bone osteonectin (BBON) and human platelet osteonectin (HPON) by N-glycanase, their ability to bind to type V collagen is increased, and HPON affinity to collagen V is the same as that of BBON. These data suggest that glycosylation of osteonectin has a direct or regulatory effect on osteonectin binding to collagen V and that the increase in tHON binding upon removal of carbohydrate is the result of a loss of a down-regulation site or direct interference of the carbohydrate at the binding site. To determine the specific role of each N-glycosylation site in tHON, Asn71 and Asn99 were mutated to Gin (N71Q, N99Q) and Thr73 and Thr101 mutated to Ala (T73A, T101A) to selectively inhibit oligosaccharide attachment. The binding affinity of N99Q and T101Q to collagen V is markedly increased over wild-type tHON, whereas N71Q and T73A are the same as wild-type tHON. The double mutant (N71Q, N99Q) binds identically to collagen V as N99Q and T101A. These data suggest that only the position 99 glycosylation site (Asn99-X-Thr101) in tHON is important in the reduction of binding of osteonectin to collagen V. Consistent with the binding data is the observation that both the N71Q and T73A mutant proteins migrate on SDS-polyacrylamide gel electrophoresis gels identically to wild-type tHON, suggesting that there is little or no N-glycosylation of residue 71 in wild-type osteonectin.

Osteonectin (ON), a single chain acidic, Ca\(^{2+}\)-binding glycoprotein, was initially identified as an integral component of the noncollagenous matrix of bone (1–3). Later studies indicated that it occurs widely in extracellular matrices and some other body compartments, as well as within cells including blood platelets (4, 5). As the endothelial and endodermal cell product it has been called SPARC (6) and as protein BM-40 obtained from the matrix of a basement membrane mouse tumor (7). It is now apparent, from DNA sequencing analyses of human (8, 9) and bovine (10) ON that the mature protein consists of 286 or 287 amino acids, respectively, and contains two potential Asn-X-Thr/Ser N-glycosylation sites, located at positions 71 and 99. The amino acid sequence of mouse SPARC is 92% identical to the cDNA-derived amino acid sequence of human ON but lacks the potential N-glycosylation site at human position 71 (11).

Structural analysis of osteonectin/BM-40/SPARC indicated that it consists of four distinct domains with potentially different ligand binding properties (10, 12, 13). Earlier studies indicated that bovine bone osteonectin binds to types I, III, and V collagen (1, 2, 14). SPARC from mouse parietal yolk sac cells binds to types III and V collagen (15). BM-40 from the mouse Engelbreth-Holm-Swarm tumor binds to type IV collagen but shows markedly reduced binding to types I, III, V, and VI collagen (16). The binding site of BM-40 for type IV collagen has been located at the EF hand and the \(\alpha\)-helical domains in the carboxyl-terminal half of BM-40 (17). Both the amino-terminal Glu-rich domain and two potential EF hands in the carboxyl-terminal half of the molecule have been implicated in Ca\(^{2+}\) binding. Early studies with proteolytic fragments indirectly suggested Ca\(^{2+}\) binding sites in carboxyl-terminal domains III and IV (16). A more recent report (17) using deletion and site-specific mutagenesis confirms this earlier finding. Osteonectin also binds specifically to plasminogen and enhances the conversion of plasminogen to plasmin by tissue plasminogen activator and may play a role during tissue remodeling or repair (18).

Kelm and Mann previously demonstrated that bone (M, 31,000) and platelet (M, 33,000) osteonectin differ in apparent molecular weight when analyzed on SDS-polyacrylamide gels (5). Subsequent studies demonstrated that the differences in mobility can be attributed to differences in N-glycosylation (14). In the same study, a comparison of the binding capacity of bone and platelet osteonectin for immobilized collagen revealed that platelet osteonectin had no apparent affinity for collagen in a concentration range in which bone osteonectin binding was observed (14). Osteoblast- and megakaryocyte-derived mRNA-encoding osteonectin are identical in size and restriction enzyme fragmentation pattern, thus lending further support to the hypothesis that differences in structure and collagen binding between bone and platelet-derived osteonectin reside at the level of N-glycosylation (19).

Full-length human osteonectin (BM-40) has been expressed in stably transfected human kidney 293 cells (20). The purified protein was very similar to mouse Engelbreth-Holm-Swarm tumor derived BM-40 in its structural and functional properties, including glucosamine and galactosamine content (20). We have now constructed for expression in 293 cells a SV40/adenovirus-derived expression vector containing the leader peptide and amino-terminal half of truncated human bone osteonectin (amino acids −17 to +146) followed by a termination

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The abbreviations used are: ON, osteonectin; tHON, recombinant truncated human osteonectin; BBON, bovine bone osteonectin; HPON, human platelet osteonectin; N-glycanase, peptide-N\(^{-}\) (N-acetyl-\(\beta\)-glucosaminyl)-asparagine amidase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; ELISA, enzyme-linked immunoassay; BSA, bovine serum albumin; TBS, Tris-buffered saline; t-PA, tissue-type plasminogen activator.
Enzymatic Removal of Oligosaccharide—Enzymatic removal of osteonectin carbohydrate was as described by Kelm and Mann (14). N-Glycanase (5 µL, 125 units) was added to individual reaction mixtures containing 20 µg of bovine bone, human platelet, or truncated human osteonectin in 50 µl of 150 mM NaCl, 20 mM Tris-HCl pH 7.6, 0.5% SDS, 50 mM β-mercaptoethanol, 50 mM o-phosphonitride, and 12.5% v/v Nonidet P-40 and incubated overnight (18 h) at 37 °C. In all experiments, samples without enzyme were treated identically to samples containing N-glycanase. After incubation, the reaction was stopped by boiling for 5 min and the detergents were removed with a microcentrifuge concentrator—30 or 10 (Amicon) column using TBS buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4). The degree of oligosaccharide removal was assessed by SDS-PAGE (8–18% gradient gel).

Construction of Wild-type Truncated Human Osteonectin Expression Vector— and Site-directed Mutagenesis—A wild-type truncated human osteonectin cDNA expression vector consisting of flanking BamHI restriction endonuclease sites, 5′-nondcoding nucleotides followed by codons for a 17 aa signal peptide and 146 NH2-terminal amino acids was amplified from aXg11 Saos-2 human osteonectin cDNA expression library clones (9) by the polymerase chain reaction (PCR) using primers 5′-CCGGCGGATCCTAT-19 nucleotide-CCGCGGATCCTAT-19 nucleotide-AG-19 nucleotide-AG-19 nucleotide-TGCCAGCAATGACAACAAGgCCTTCGACTCT-3′ (inverse complement). The PCR product was subcloned into the BamHI site of the mammalian cell expression vector pDS (22) and sequenced in its entirety. Site-directed mutagenesis to abolish potential N-glycosylation sites in THON was performed by the PCR-mediated method developed in this laboratory (21) using linearized (Stul-digested) wild-type THON/pDS expression vector as template. As revealed by sequencing, the expression vector contained the following mutations: primer B, 5′-ggtgtactagtaacctaggAGCCATACCACATTTGTAGAGGT-3′, a 5′-I restriction site; and primer D, 5′-TGCGAGCTCCTAT-19 nucleotide-TGCCAGCAATGACAACAAGgCCTTCGACTCT-3′. The PCR-amplified products were verified by the addition of 100 µl of TBS buffer (100 mM NaCl, 10 mM Tris, pH 7.4) to an equal volume of 2% BSA in TBS/CaCl2 for 1 h at room temperature, followed by blocking and washing. Different concentrations of osteonectin were measured by reaction was carried out in triplicate and showed a coefficient of variation in the range 0.4–1.8%.

Affinity of Antibody IIIA3A8 for Different Osteonectins—Modifications of an ELISA method based on the supplier’s instruction (Hematologic Technologies Inc., model 373A DNA sequence) was used to confirm the expected affinities and to exclude inadvertent mutations introduced from PCR. Sequencing reactions were performed with the TaqDye Deoxy® terminator cycle sequencing kit (ABI) according to the supplier’s instructions.

Cell Culture and Transfection—Human kidney 293 cells were cultured in Dulbecco’s modified Eagle’s medium, high glucose (Life Technologies, Inc., product number 11995-016) supplemented with 10% fetal bovine serum and grown to 80–90% confluence. 20–40 µg of wild-type THON or mutant expression vector was transfected into human kidney 293 cells by the calcium phosphate precipitation method (23, 24). The plate (10 cm diameter) was washed twice with Tris-buffered saline after 4 h transfection and cultured in 10 ml of Dulbecco’s modified Eagle’s medium without serum for 48 h. The serum-free conditioned medium was then collected for purification of recombinant protein. Typically, conditioned media contained 10–20 µg/ml of recombinant protein. The levels for N71Q were, however, significantly lower (data not shown).

Purification of Recombinant Protein—Slight modification of the method described elsewhere (5, 20) was used for the isolation of wild-type and mutant THON. Conditioned serum-free medium was diluted with an equal volume of 20 mM Tris-HCl, pH 7.4, buffer and adjusted to 5 mM EDTA and 5 mM benzamidine. The diluted conditioned medium was applied to a 5-ml DEAE-Sephadex A-25 (Sigma) column equilibrated in the same buffer and washed with the above buffer until the medium pigment eluted from the column. The column was then eluted with a 0.2–0.8 M NaCl gradient in 20 mM Tris-HCl, pH 7.4, 5 mM EDTA buffer. Fractions were collected and analyzed by SDS-PAGE followed by silver staining (25). Wild-type THON and mutants eluted in a narrow range around 0.4 M NaCl. Fractions containing the desired protein were applied directly at a flow rate of 0.4 ml/min onto an anti-ON monoclonal antibody (IIIA4) affinity column equilibrated in 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 4 °C. The column was washed with the same buffer and recombinant osteonectin eluted with 5 ml of 1 M NaCl, 20 mM Tris-HCl, pH 7.6. The isolated protein was concentrated on a Centricon-10 column (Amicon, Inc., Beverly, MA) according to the manufacturer’s instruction. Protein concentration was determined by the micro-BCA protein assay (Pierce) following the supplier’s instruction and using bovine bone osteonectin as a standard, and purity was assessed by 8–18% gradient SDS-PAGE followed by Coomassie Blue staining.

Peptide-N-glycanase (5 µL, 125 units) was added to individual reaction mixtures containing 20 µg of bovine bone, human platelet, or truncated human osteonectin in 50 µl of 150 mM NaCl, 20 mM Tris-HCl pH 7.6, 0.5% SDS, 50 mM β-mercaptoethanol, 50 mM o-phosphonitride, and 12.5% v/v Nonidet P-40 and incubated overnight (18 h) at 37 °C. In all experiments, samples without enzyme were treated identically to samples containing N-glycanase. After incubation, the reaction was stopped by boiling for 5 min and the detergents were removed with a microcentrifuge concentrator—30 or 10 (Amicon) column using TBS buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4). The degree of oligosaccharide removal was assessed by SDS-PAGE (8–18% gradient gel).
RESULTS

Affinity of Antibody IIIA3A8 to Different Forms of Osteonectin—Fig. 1 demonstrates that over a concentration range of 5–100 nM osteonectin, the affinity and capacity for monoclonal antibody IIIA3A8 is equivalent for both bovine bone- and human platelet-derived ON, as well as for the recombinant truncated wild-type and mutated species. These results validate the use of IIIA3A8 to detect the different forms of osteonectin in the binding assays described in this study.

Binding of tHON to Type V Collagen—Fig. 2 shows the binding of both tHON and BBON to type V collagen immobilized on microtiter wells. The results indicate that tHON containing amino acids 1–146 binds to type V collagen to nearly the same extent and same affinity (half-maximum binding at \(-0.07\) μM) as full-length bovine bone osteonectin.

Binding of N-Glycanase-treated Osteonectin to Type V Collagen—After enzymatically removing N-linked oligosaccharide chains from BBON, HPON, and tHON with N-glycanase, each sample showed an increase in electrophoretic mobility corresponding to a 4–5-kDa decrease in mass (Fig. 3). It is also clear from these results that the antibody IIIA3A8 does not require N-linked oligosaccharide in its epitope. Enzymatic removal of N-linked carbohydrate from both BBON and tHON results in a 2-fold increase in type V collagen binding capacity (Fig. 4). The results presented in Fig. 4A also demonstrate that deglycosylated HPON is now capable of binding to type V collagen and with the same apparent affinity (half-maximum binding = 0.07 μM) as deglycosylated BBON and tHON. These data demonstrate that glycosylation of ON leads to a reduction in ON binding capacity to collagen and not affinity and that the increase in ON binding upon removal of carbohydrate is the result of a loss of either a distal down-regulation site or direct interference by carbohydrate at the binding site.

N-Glycosylation Site-directed Mutagenesis—To analyze the individual role of potential ON N-glycosylation sites in binding to type V collagen, we designed Asn to Gln mutations (N71Q, N99Q, and N71,99Q) to prevent attachment of the oligosaccharide to type V collagen, and N-glycan—Fig. 1 demonstrates that over a concentration range of 5–100 nM osteonectin, the affinity and capacity for monoclonal antibody IIIA3A8 is equivalent for both bovine bone- and human platelet-derived ON, as well as for the recombinant truncated wild-type and mutated species. These results validate the use of IIIA3A8 to detect the different forms of osteonectin in the binding assays described in this study.

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Binding of N-Glycanase-treated Osteonectin to Type V Collagen—After enzymatically removing N-linked oligosaccharide chains from BBON, HPON, and tHON with N-glycanase, each sample showed an increase in electrophoretic mobility corresponding to a 4–5-kDa decrease in mass (Fig. 3). It is also clear from these results that the antibody IIIA3A8 does not require N-linked oligosaccharide in its epitope. Enzymatic removal of N-linked carbohydrate from both BBON and tHON results in a 2-fold increase in type V collagen binding capacity (Fig. 4). The results presented in Fig. 4A also demonstrate that deglycosylated HPON is now capable of binding to type V collagen and with the same apparent affinity (half-maximum binding = 0.07 μM) as deglycosylated BBON and tHON. These data demonstrate that glycosylation of ON leads to a reduction in ON binding capacity to collagen and not affinity and that the increase in ON binding upon removal of carbohydrate is the result of a loss of either a distal down-regulation site or direct interference by carbohydrate at the binding site.

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Expression, Purification, and Properties of Wild-type tHON and Its Mutants—All constructs were transiently expressed in cultured human kidney 293 cells and were purified as described under “Experimental Procedures.” Amino acid sequencing of isolated wild-type tHON revealed that the 17-amino acid leader peptide had been properly removed prior to secretion (data not presented). Each reduced sample shows a single broad band upon 8–18% SDS-PAGE and Coomassie Blue staining. Lane 1, the size of molecular mass standards is indicated in kilodaltons; lanes 2 (HPON), 3 (BBON), and 6 (tHON) represent the untreated proteins. Lanes 3 (HPON), 5 (BBON), and 7 (tHON) represent the enzyme-treated proteins. Western blotting with antibody IIIA3A8 also resulted in visualization of the above bands (data not shown).
that of wild-type tHON and suggest that there is little or no glycosylation of residue 71 in wild-type tHON. This interpretation is also consistent with the gel mobility data presented in Fig. 5. Fig. 6B, in contrast, shows that mutation of residue 99 or 101 dramatically increases the binding of ON to collagen and in a manner similar to that resulting from enzymatic deglycosylation of wild-type tHON (Fig. 4B). Fig. 6B also shows that the double mutant, N71,99Q, has nearly identical binding to collagen as either N99Q or T101A, further indicating that the effect of glycosylation on collagen binding is solely due to modification of osteonectin residue 99.

**DISCUSSION**

Enzymatic deglycosylation studies suggest that bone osteonectin possesses a high mannose-type oligosaccharide structure, whereas platelet osteonectin likely possesses a complex-type structure (14) and may be due to differential expression of glycosyltransferases by osteoblasts and megakaryocytes (26, 27). Based upon differences in glycosylation patterns as well as lectin binding properties, it was proposed that the collagen binding specificity of bone and platelet osteonectin is related to differences in glycosylation (14). In the present study we demonstrate that after removal of oligosaccharide chain structures from bovine bone and human platelet osteonectin by N-gly-

**FIG. 5. Wild-type tHON and N-glycosylation mutants.** Wild-type tHON, N71Q, T73A, N99Q, T101A, and N71,99Q (3 μg) following immunoaffinity purification were run on an 8–18% SDS-PAGE gradient gel and visualized by staining with Coomassie Blue. Lane 1, sizes of protein standards are indicated in kilodaltons; lanes 2 and 8, tHON; lane 3, N71Q; lane 4, T73A; lane 5, N99Q; lane 6, T101A; and lane 7, N71,99Q. Immunoblotting revealed identical bands as shown in the figure (data not presented).

**FIG. 6. Binding of N-glycosylation mutants to type V collagen.** Samples were prepared as described under "Experimental Procedures" and assayed as described for Fig. 4. A, wild-type tHON (○), N71Q (●), and T73A (▲). B, wild-type tHON (○), N99Q (▲), T101A (□), and N71,99Q double mutant (■).
binding to collagen and that the glycosylation pattern dramatically affects binding to collagen. In addition, studies with a truncated form of human osteonectin containing only the amino-terminal half of the protein effectively binds to type V collagen, demonstrating that the binding region of ON resides within amino acid residues 1-146. As in the case of BBON and HPON, N-glycanase treated truncated ON displays increased binding to type V collagen.

In order to further define the region of ON involved in collagen binding and the effects of specific N-glycosylation sites on biological activity, we have systematically altered, by site-specific mutagenesis, each of the two potential Asn-X-Thr glycosylation sites in osteonectin. Both sites are within the amino-terminal half of the mature protein, affording the use of the truncated ON cDNA as template for mutagenesis as well as for production of wild-type counterpart recombinant protein. Expression vectors for wild-type hON, one double and four single mutants of hON (N71,99Q and N71Q, T73A, N99Q, T101A) were constructed using the PCR-enabled method of Nelson and Long (21). Each expression vector was then used to transiently express recombinant protein in human kidney 293 cell cultures, and secreted protein was subsequently purified from conditioned media and characterized.

Initial studies revealed that SDS-PAGE migration of both the N99Q and T101A mutant recombinant proteins is identical and significantly faster than wild-type protein, suggesting a similar reduction in glycosylation for both mutants. Estimates based on mobility differences suggest a loss of about 4-5 kDa mass for both mutants. The mass difference is similar to that observed by Pottgiesser et al. (17) when the protein was expressed in the presence of tunicamycin or treated with N-glycosidase F. Surprisingly, both the N71Q and T73A mutants migrated identically to wild-type protein, suggesting that there is little or no N-glycosylation in the wild-type protein at this position. Consistent with these results is the observation that the N71,99Q double mutant migrates identically to the N99Q and T101A single mutant proteins. The interpretation of little or no glycosylation at human position 71 is also consistent with the report of Nischt et al. (20), indicating that mouse tumor-derived osteonectin (BM-40) lacking a potential N-glycosylation site at residue 71 and recombinant human osteonectin have the same carbohydrate content and composition.

Differential glycosylation at the two potential N-glycosylation sites suggested by the above structural analysis is also implicated by functional studies relating to type V collagen binding. Data presented above indicate that mutation at either residue 71 or 73 has no effect on collagen binding compared with either wild-type hON or BBON. However, mutation at residue 99 or 101, thereby abolishing N-glycosylation at residue 99, results in protein with significantly enhanced collagen binding and are consequentially masked by the presence of N-linked carbohydrate at this position. Alternatively, N-glycosylation of residue 99 may have an indirect distal allosteric regulatory effect.

Saturation binding curves presented in this communication suggest that the presence of N-linked carbohydrate decreases the capacity of osteonectin for type V collagen but does not have a measurable effect on binding affinity. Under the conditions of the binding assay used in our studies, all species of osteonectin achieved half-maximum binding at 0.07-0.10 μM, which is close to the apparent dissociation constant (Kd = 0.2 μM) for bovine bone osteonectin derived by Kelm and Mann from a Scatchard plot (14).

The negative effect of N-linked carbohydrate on ligand binding is not unique to osteonectin. Grinnell et al. (28), for example, report that abolition of an N-glycosylation site in protein C by mutagenesis (N313Q) results in a protein having about a 3-fold decrease in apparent Kd (6.1 μM → 1.9 μM) for thrombin-thrombomodulin activation. Wittwer et al. (29) observed a 3-4-fold reduction in fibrin binding and clot lysis activity by tissue-type plasminogen activator (t-PA) synthesized by Bowes melanoma and human colon fibroblasts in the presence of tunicamycin. Similar results for t-PA were obtained with tunicamycin in Chinese hamster ovary cells by Hanssen et al. (30). In the latter report, mutation of all three potential Asn N-glycosylation sites in t-PA also resulted in a marked increase in fibrin binding (30). Lijnen et al. (31) have also reported that the apparent dissociation constant for binding of plasminogen to its natural protein inhibitor α2-antiplasmin is affected by the presence of N-linked carbohydrate. Both Glu- and Lys-plasminogen Kd values (4.2 and 1.9 μM, respectively) are reduced upon removal of carbohydrate (2.9 and 0.2 μM, respectively).

Type V collagen is particularly abundant in vascular tissue, primarily due to synthesis in smooth muscle cells (32). Smooth muscle cells and their protein products are believed to play an important role in the development of atherosclerotic plaque. The ratio of type V collagen to other types is increased in human atherosclerotic lesions (33, 34). In addition to its possible role in atherosclerosis, type V collagen may also be important in hemostasis. It has been reported to not stimulate platelet aggregation (35) and prevent platelet adhesion (36) and consequently would have an anti-thrombotic effect. This effect may be mediated through its binding to the cytoadhesive molecule thrombospondin (37). Recently a 19-kDa fragment from bovine thrombospondin has been identified which binds to both type V collagen and heparin (38). Type V collagen has been reported to also inhibit the activation of the intrinsic clotting path factor XII (39).

On the other hand, procoagulant activity by type V collagen has also been reported (40). Coating of vascular prostheses surfaces with type V collagen inhibits endothelial cell attachment and growth (41) and as a result may also contribute to a prothrombotic state. A similar prothrombotic state, due to the absence of endothelial cells, may exist at sites of tissue injury and remodeling. Kelm et al. have recently reported that osteonectin also binds to plasminogen and enhances t-PA conversion of plasminogen to plasmin (18). Both plasminogen and t-PA are important antithrombotic agents. The authors also reported the mediation of plasminogen binding to type V colla-
tion in the absence of functional endothelium. Consequently, osteonectin, by serving as a bridge between exposed type V collagen and antithrombotic agents such as plasminogen and t-PA, may play an important compensatory role in preventing unwanted clot formation in the absence of functional endothelium.

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