αvβ3 and αvβ5 Integrins Bind Both the Proximal RGD Site and Non-RGD Motifs within Noncollagenous (NC1) Domain of the α3 Chain of Type IV Collagen

IMPLICATION FOR THE MECHANISM OF ENDOTHELIAL CELL ADHESION*

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Abstract

The NC1 domains of human type IV collagen, in particular α3NC1, are inhibitors of angiogenesis and tumor growth (Petitclerc, E., Boutaud, A., Prestayko, A., Xu, J., Sado, Y., Ninomiya, Y., Sarras, M. P., Jr., Hudson, B. G., and Brooks, P. C. (2000) J. Biol. Chem. 275, 8051–8061). The recombinant α3NC1 domain contained a RGD site as part of a short collagenous sequence at the N terminus, designated herein as RGD-α3NC1. Others, using synthetic peptides, have concluded that this RGD site is nonfunctional in cell adhesion, and therefore, the antiangiogenic activity is attributed exclusively to αvβ3 integrin interactions with non-RGD motifs of the RGD-α3NC1 domain (Maeshima, Y., Colorado, P. C., and Kalluri, R. (2000) J. Biol. Chem. 275, 23745–23750). This nonfunctionality is surprising given that RGD is a binding site for αvβ3 integrin in several proteins. In the present study, we used the α3NC1 domain with or without the RGD site, expressed in HEK 293 cells for native conformation, as an alternative approach to synthetic peptides to assess the functionality of the RGD site and non-RGD motifs. Our results demonstrate a predominant role of the RGD site for endothelial adhesion and for binding of αvβ3 and αvβ5 integrins. Moreover, we demonstrate that the two non-RGD peptides, previously identified as the αvβ5 integrin-binding sites of the α3NC1 domain, are 10-fold less potent in competing for integrin binding than the native protein, indicating the importance of additional structural and/or conformational features of the α3NC1 domain for integrin binding. Therefore, the RGD site, in addition to non-RGD motifs, may contribute to the mechanisms of endothelial cell adhesion in the human vasculature and the antiangiogenic activity of the RGD-α3NC1 domain.

Type IV collagen is the major constituent of basement membranes, a specialized form of extracellular matrix underlying all epithelia, that compartmentalizes tissues and provides molecular signals for influencing cell behavior. The type IV collagen family is comprised of six α-chains (α1–α6) that assemble into three kinds of triple-helical protomers of different chain composition. Each protomer has three functional domains: a 7 S domain at the N terminus, a long triple-helical collagenous domain in the middle of the molecule, and a trimeric noncollagenous (NC1) domain at the C terminus. Protomers self-assemble into networks by end-to-end associations that connect four 7 S domains at one end and connect two NC1 trimeric domains at the other end, forming an NC1 hexamer configuration (1). Three types of networks are known: an α1α1α2 network, present in the basement membranes of all tissues and animal phyla and α3α4α5 and α1α2α5α6 networks that have a restricted tissue distribution. These networks are essential for tissue development and function. They provide mechanical stability, a scaffold for assembly of other macromolecular components, and act as a ligand for integrins, receptors that mediate cell adhesion, migration, growth, and differentiation.

Cell adhesion to the ubiquitous α1α1α2 (IV) network has been demonstrated for a variety of cell types (2–4), including endothelial (5, 6) and tumor cell lines (7, 8). It is mediated by integrin binding to both triple-helical and NC1 domains. Specifically, integrins αvβ3 and αvβ5 were identified as major receptors for the collagenous domain (9), and their binding sites have been subsequently mapped (10, 11). Additional integrins, such as αvβ1, that bind the triple-helical domain may be involved (12, 13). The NC1 domain was initially characterized as a ligand for αvβ3 and αvβ5 integrins in human mesangial cells (4), and binding of αvβ3 integrin to recombinant α1NC1 was later confirmed (14). In contrast, recombinant α2NC1 was identified as a novel ligand for a different subset of integrins (αvβ3, αvβ5, and αvβ6) in endothelial cells, suggesting the existence of a non-RGD-binding motif (15).

Cell adhesion to the α3α4α5(IV) network is less understood because only the recombinant NC1 domains are available for study. It is interesting that the individual human NC1 domains, expressed in mammalian cells, have strikingly different effects on endothelial cells. The α3NC1 domain strongly activates both adhesion and migration, whereas the α4NC1 and α5NC1 domains are inactive (15), despite high sequence homology among all three NC1 domains, suggesting that the α3NC1 domain contains unique structural determinants mediating these effects. Experiments with neutralizing antibodies provided the first evidence that endothelial cell adhesion to α3NC1 domain was mediated by αvβ3 integrin (15). In these studies, the recombinant protein contained a RGD site within a 12-residue collagenous sequence proximal to the α3NC1 domain.
Integrin Binding to the α3NC1 Domain of Type IV Collagen

In this report this recombinant protein is expressed as RGD-α3NC1 to emphasize the presence of the RGD site. Cell adhesion to the RGD-α3NC1 domain could be mediated by αβ3 binding to the RGD sequence, a well known integrin-binding site in numerous proteins, or to non-RGD motifs within the α3NC1 domain. In a subsequent study, Maeshima et al. (16) showed that this RGD site of the RGD-α3NC1 domain, termed tumstatin, was nonfunctional in cell adhesion and concluded that it does not bind αβ3 integrin. Instead, they identified a non-RGD region comprising residues 54–132 of the α3NC1 domain that bound αβ3 integrin, which was later narrowed down to 25 residues using deletion mutagenesis and synthetic peptides (17). Another non-RGD region of the α3NC1 domain, residues 185–203, identified by Han et al. (18) was demonstrated to inhibit proliferation of melanoma cells, and the receptor for this synthetic peptide was identified as αβ3 integrin by affinity chromatography (19). Whether these two non-RGD motifs quantitatively account for the adhesive activity of the native RGD-α3NC1 domain and its capacity to bind αβ3 integrin has not been addressed.

The NC1 domains of certain α-chains of type IV collagen also display activity as inhibitors of angiogenesis and tumor growth. The capacity of the exogenous α1NC1 and α2NC1 domains to disrupt basement membrane assembly, blocking tissue development in vivo, was first described in Hydra vulgaris (20). This observation led us to evaluate the capacity of individual recombinant NC1 domains to perturb the basement membrane assembly of developing blood vessels. The α2NC1, RGD-α3NC1, and α6NC1 domains potently inhibited both angiogenesis and tumor growth in a chick chorioallantoic membrane system, with RGD-α3NC1 exhibiting the strongest effect, whereas NC1 domains of the α1, α4, and α5 chains had no effect. This inhibitory activity is presumably mediated by the αβ3 integrin binding to the RGD and/or non-RGD motifs (15). Subsequent studies have revealed that the anti-angiogenic activity of RGD-α3NC1 domain (tumstatin) is potentially associated with inhibition of cell proliferation, induction of apoptosis, and activation of caspase-3 specifically in endothelial cells (21).

Furthermore, it has been shown that both tumstatin and its non-RGD peptide inhibit cap-dependent translation only in endothelial cells by negative regulation of mTOR signaling (22, 23), implicating that the anti-angiogenic activity depends on binding of the αβ3 integrin to non-RGD motifs but not to the RGD. More recent studies lead to the supposition that the α3NC1 domain can function as an endogenous suppressor of αβ3 integrin-mediated pathologic angiogenesis and tumor growth (24). The finding that the RGD site is nonfunctional for αβ3 integrin binding, revealed with synthetic peptides, is surprising, because it is a key binding site in several matrix proteins (25, 26).

An understanding of the molecular mechanism of integrin-mediated cell adhesion of the RGD-α3NC1 domain is ultimately important given the potential role of this protein as a pharmacological and endogenous regulator of angiogenesis and tumor growth. This requires identification of the integrin receptors, recognition sites within RGD-α3NC1 domain, and the ligand contact points within the integrin. In the present study, an alternative to the synthetic peptides approach was used to assess the functionality of the RGD and non-RGD motifs in the context of native protein conformation. This was accomplished by using recombinant proteins/chimeras with and without the RGD sequence, expressed in mammalian cells to ensure native conformation, for endothelial cell adhesion and integrin binding assays. Our results provide unambiguous evidence that both the RGD and non-RGD motifs bind αβ3 integrin and mediate the adhesion of endothelial cells to the RGD-α3NC1 domain. These findings suggest that both motifs may contribute to the anti-angiogenic activity of the RGD-α3NC1 domain.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal antibodies Mab-3 to α3NC1 were purchased from Wieslab AB (Lund, Sweden). Goodpasture autoantibodies were purified from GP human serum by affinity chromatography on protein A-agarose. Purified αβ3, αβ4, αβ6, and αβ1 integrins and monomeric integrin antibodies LM609 (anti-αv, β3) and P1F6 (anti-αv, β6) were from Chemicon (Temecula, CA). Integrin monoclonal antibodies P2W7 (anti-αv) and 4B7R (anti-β3) were from Santa Cruz (Santa Cruz, CA); monoclonal antibodies AIIIB2 (anti-β3) and BIIG2 (anti-αv), developed by Dr. Caroline H. Damsky, were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). M2 monoclonal antibodies to FLAG peptide, RGDS peptide, and fibronectin were from Sigma; vitronectin was from TakRa Biomicals (Shiga, Japan). Synthetic peptides T3 (LQRFTMPFLCNVNDVCNF) and 185–203 (CNYSSNSYFLASLNP) were purchased from Multiple Peptide Synthesis (San Diego, CA) and SynPep Corp. (Dublin, CA), respectively.

Cell Culture—Human umbilical vein endothelial cells (HUVEC) were obtained from BioWhittaker (Charlestown, NC). The cells were grown in EGM-2 MV medium (BioWhittaker) and used between passages 3 and 7.

Proteins—Recombinant human NC1 domains of type IV collagen that carried the FLAG sequence on the N terminus were stably expressed in HEK 293 cells and purified from conditioned medium by affinity chromatography on anti-FLAG agarose as described previously (27). α3NC1 domain was created as a deletion mutant of RGD-α3NC1 lacking 12 amino acid residues from the N terminus by PCR using RGD-α3/pRC/CMV vector expression as a template and the following primers: 5‘-ATA TGC TAG CTT CAA CCA CCT GGA CAA CGA GAG (forward) and 5‘-CAG CGA GCT CTA GCA GTA TTT AGG (reverse). Purified PCR product was digested with NheI and Apal restriction enzymes and subcloned into the pRc/CMV vector for protein expression. Prior to transfection in HEK 293 cells, the α3NC1 insert was sequenced in both directions to verify the sequence.

Cell Adhesion Assay—Proteins in TBS buffer or synthetic peptides in 50 mM NaCO3/NaHCO3 buffer, pH 9.5, were immobilized on 96-well plates (Nunc, Rochester, NY) at 4 °C overnight. Nonspecific binding sites were blocked with 1% BSA in TBS for 2 h at 30 °C, and the wells were washed twice with TBS. Subconfluent HUVEC were harvested, washed, and resuspended in adhesion buffer containing Ham’s F-12/Dulbecco’s modified Eagle’s medium, 1 mM MgCl2, 0.2 mM MnCl2, and 0.5% BSA. 5 × 104 cells were added to each well and allowed to attach for 60 min at 37 °C in a CO2 incubator. In some experiments, the cells were pretreated for 30 min with integrin-specific antibodies or peptides prior to their addition to the wells. After removal of the nonadhered cells by washing with TBS, the attached cells were fixed and stained with 0.1% crystal violet as described (28). The wells were washed three times with TBS, and cell-associated crystal violet was eluted by the addition of 100 μl of 10% acetic acid. Cell adhesion was quantified by measuring the absorbance of eluted dye at 595 nm with a microtiter reader. All of the presented data were corrected for background binding in blank wells blocked with BSA.

Cell Membrane Labeling—HUVEC were grown in EGM-2 MV medium, detached from culture dishes with 2 mM EDTA in Hanks’ balanced salt solution, and collected by centrifugation for 5 min at 800 × g. After two washes with cold phosphate-buffered saline, the cells were resuspended in phosphate-buffered saline at 1 × 107 cells/ml. Sulfo-NHS-biotin (Pierce) was added to a final concentration of 100 μg/ml and incubated with cells for 1 h at room temperature with gentle mixing. The cells (1 × 107) were washed three times with cold phosphate-buffered saline, 1 mM MgCl2, 1 mM MnCl2, 1 mM phenylmethylsulfonyl fluoride and 40 μg/ml aprotinin for 4 min at 4 °C with TBS containing 100 mM octylglucoside, 1 mM CaCl2, 1 mM MgCl2, 1 mM MnCl2, with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.2 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin). The supernatant was collected after centrifugation for 30 min at 16,000 × g and stored at −70 °C.

Affinity Chromatography—Purified recombinant RGD-α3NC1 (1 mg of resin) was coupled to the Affi-Gel 10 (Bio-Rad) in 0.1 M MOPS buffer, pH 7.0. The remaining active groups were blocked with 0.1 M diethanolamine. Coupling efficiency was 75% as determined by absorbance

2 The abbreviations used are: GP, Goodpasture; BSA, bovine serum albumin; HUVEC, human umbilical vein endothelial cells; TBS, Tris-buffered saline; MOPS, 4-morpholinosopropanesulfonic acid; NC1, the noncollagenous domain.
incubated with washing buffer (TBS, 1 mM MgCl₂, 0.2 mM MnCl₂, 0.01% Tween 20) and incubated for 2 h at 30 °C.

identical to those in H9251 inhibitors. Biotinylated HUVEC extract was loaded on the RGD-3NC1 column and incubated for 60 min. The column was washed with washing buffer and eluted with 10 mM EDTA in TBS, 50 mM octylglucoside with protease inhibitors. 0.5-ml fractions were collected, and 20-μl aliquots were tested for the presence of biotin by direct enzyme-linked immunosorbent assay using streptavidin-horseradish peroxidase conjugate, and visualized by enhanced chemiluminescence (Pierce).

Immunoprecipitation/Western Blotting—Protein A/G-agarose was preabsorbed with unlabeled HUVEC protein extract prepared as described above. Aliquots of dialyzed fraction eluted from RGD-3NC1 affinity column were preincubated with integrin antibodies in immunoprecipitation buffer (TBS, 1 mM MgCl₂, 0.5% Nonidet P-40, 0.1% BSA) for 2 h at 4 °C followed by incubation with protein A/G-agarose beads for 5 h at 4 °C. The beads were washed once with immunoprecipitation buffer and four times with modified RIPA buffer (TBS, 1% Nonidet P-40, 0.5% deoxycholate). Immunoprecipitated proteins were run on 6% SDS-PAGE, transferred to nitrocellulose membranes, incubated with streptavidin-horseradish peroxidase conjugate, and visualized by enhanced chemiluminescence (Pierce).

Solid Phase Ligand Binding Assay—Microtiter plates were coated with various proteins and blocked with 1% BSA/TBS as described for cell adhesion assay. Purified integrins were overlaid in binding buffer (TBS, 0.1% BSA, 1 mM MgCl₂, 0.2 mM MnCl₂, 50 mM octylglucoside) and incubated for 2 h at 30 °C. The plates were washed three times with washing buffer (TBS, 1 mM MgCl₂, 0.2 mM MnCl₂, 0.01% Tween 20) and incubated with αᵣ integrin antibodies (P2W7, 1:500) for 1 h. After extensive washes, the bound antibodies were detected using alkaline phosphatase-conjugated anti-mouse IgG antibodies. p-Nitrophenyl phosphate substrate (Sigma) was added to the wells, and absorbance was measured at 410 nm. Nonspecific binding obtained by preincubation of purified integrins with 10 mM EDTA for 30 min at 4 °C was subtracted from all of the obtained values.

**Statistical Analysis**—The data are expressed as the means ± S.D., and statistical analysis was performed using Student’s t test for unpaired samples. Differences were considered statistically significant if the p values were less than 0.05.

**RESULTS**

Experimental Strategy and Expression of Recombinant NC1 Domains/Chimeras—In our earlier studies, the human α3NC1 domain was expressed as a recombinant protein containing a 12-residue collagenous sequence at the N terminus, as a strategy to map the locations of epitopes for GP autoantibodies (29, 30). The entire sequence was required to match that of the native fragment, produced by collagenase digestion of native basement membranes, to ensure the preservation of epitopes (31). Subsequently, we used this protein in studies of cell adhesion, migration, tumor growth, and angiogenesis (15). This recombinant protein, equivalent to tumstatin (NCBI accession number AAF72632) in other reports (16, 21), is designated herein as RGD-α3NC1 (Fig. 1A) to denote the presence of the RGD site in the short collagenous sequence and to distinguish it from protein containing only the α3NC1 domain. Likewise, α1/α3 chimera, composed of the α1NC1 domain along with the
Although cell adhesion to the motifs of the NC1 domain in endothelial cell adhesion and integrin binding. To do this, we expressed the α3NC1 with and without the N-terminal RGD sequence and the α1NC1 domain with and without RGD site from α3 chain. Recombinant NC1 domains and chimeras (Fig. 1A) were expressed in HEK 293 cells to ensure proper folding and disulfide bond formation, as demonstrated in our previous studies defining conformational epitopes for GP antibodies (29, 30). The migration patterns of purified RGD-α3, RGD-α1, and α3NC1 domains on SDS-PAGE were in agreement with their expected molecular masses (28.4, 28.3, and 27.2 kDa, respectively). Moreover, the RGD-α3NC1 and α3NC1 domains were immunologically identical when checked by Western immunoblotting with conformational-dependent GP and Mab-3 antibodies (Fig. 1B), confirming that they were properly folded.

Role of the Proximal RGD Site and NC1 Domain in Endothelial Cell Adhesion to RGD-α3NC1—Our previous data demonstrated differential activity of recombinant NC1 domains of the six α-chains of type IV collagen for endothelial cell interactions (15). In the present study, dose-response curves for cell adhesion to these domains were measured to establish a foundation for subsequent experiments (Fig. 2A). Among the NC1 domains, RGD-α3NC1 displays the strongest capacity in promoting HUVEC adhesion and spreading in a concentration-dependent and saturable manner, whereas α1NC1 has minimal activity. The adhesive activity of RGD-α3NC1 was comparable with that of fibronectin (Fig. 2B). HUVEC adhesion and spreading on RGD-α3NC1 was completely abolished by preincubation with EDTA (Fig. 2B), suggesting that the adhesion is integrin-dependent. Similar results showing preferential cell adhesion to NC1 domains on SDS-PAGE were in agreement with their expected molecular masses (28.4, 28.3, and 27.2 kDa, respectively). Moreover, the RGD-α3NC1 and α3NC1 domains were immunologically identical when checked by Western immunoblotting with conformational-dependent GP and Mab-3 antibodies (Fig. 1B), confirming that they were properly folded.

Fig. 2. Effect of recombinant NC1 domains of type IV collagen on endothelial cell adhesion. A. NC1 domains of type IV collagen (○, α1; ▽, α2; ▪, RGD-α3, ▼, α4; ■, α5; □, α6) were coated on 96-well plates, and HUVEC adhesion was determined after incubation for 1 h at 37 °C. Coating efficiency of all NC1 domains determined by enzyme-linked immunosorbent assay with anti-FLAG antibody was essentially equal. The data points represent the mean absorbance ± S.D. of triplicate wells. B. Phase contrast photographs of HUVEC after incubation for 1 h at 37 °C on wells coated with BSA (panel a), 10 μg/ml of fibronectin (panel b), or 20 μg/ml of RGD-α3NC1 in the absence (panel c) or the presence of 10 mM EDTA (panel d) (original magnification 200×). Note the round morphology of nonattached cells in panels a and d.

The presence of the RGD sequence is a unique feature of RGD-α3 when compared with all other NC1 domains. To explore the functionality of the RGD site, as well as the non-RGD motifs within the α3NC1 domain, we constructed recombinant chimeras for gain- and loss-of-function. As shown in Fig. 3A, the RGD-α3 and α3NC1 domains were capable of supporting HUVEC adhesion in a dose-dependent and saturable manner; however, the α3NC1 was only 54% as active as RGD-α3NC1. Such a decrease upon the removal of the RGD site from RGD-α3NC1 directly demonstrates the functional role of RGD in cell adhesion. In the case of the α3NC1 domain, which has no RGD site, the remaining cell adhesion is clearly conferred by non-RGD motifs. To further support the role of the RGD site, we used the α1NC1 domain and RGD-α1NC1 chimera (Fig. 3B). Although cell adhesion to the α1NC1 domain was minimal, the introduction of a RGD site in RGD-α1NC1 strongly increased its HUVEC adhesion activity to a level higher than α3NC1, albeit not to that of RGD-α3NC1 (Fig. 3B). Thus, our results demonstrate the activity of both the RGD site and the non-RGD motifs of the NC1 domain in cell adhesion to RGD-α3NC1 by the gain-of-function (RGD-α1NC1) and loss-of-function (α3NC1) approaches. Moreover, our data indicate that the RGD site plays a dominant role in cell adhesion.

Identity of HUVEC Integrin Receptors for the RGD-α3NC1 Domain—To determine the identity of integrins that may bind to the RGD-α3NC1 and act as mediators of endothelial cell adhesion, we used the direct approach of affinity chromatography. HUVEC membrane proteins were labeled with an impermeable biotin label and solubilized with octylglucoside, and the lysate was applied to a RGD-α3NC1 column. Immunoprecipitation of the EDTA eluate from an affinity column with specific antibodies revealed the presence of αβ2 and smaller amounts of αβ5 integrin heterodimers (Fig. 4A). This suggests either a higher affinity of αβ2 integrin toward the RGD-α3NC1 when compared with αβ5 or alternatively, it may result from a somewhat lower expression level of αβ2 integrin (Fig. 4B). Molecular masses of integrin heterodimers under nonreducing conditions were about 160 (α5) and 95 (β3 and β5) kDa, which are in agreement with those reported by others (32, 33). With the exception of αβ6, other integrins including αβ1, αβ3, and αβ2 were also abundantly expressed in endothelial cells (Fig. 4B) but were not detected in the fraction eluted with EDTA from affinity column. These results demonstrated a specific interaction between αβ3 and αβ2 integrins and the RGD-α3NC1 domain.

Relative Contributions of αβ2 and αβ5 Integrins to Cell Adhesion—To determine the functional significance of αβ2 and αβ5 integrin binding to the RGD-α3NC1 domain in endothelial cell adhesion, we examined the effect of integrin blocking antibodies. Cell adhesion to the RGD-α3NC1, RGD-α1NC1, and α3NC1 domains was strongly inhibited with αβ2 blocking antibodies (Fig. 5A). Surprisingly, αβ5 antibodies had no inhibitory effect, either alone or in combination with αβ2 antibodies, suggesting that αβ2 plays a minor role, if any, in endothelial cell adhesion to the RGD-α3NC1 domain. Neutralizing antibodies to α5 and β3 integrin subunits blocked HUVEC adhesion to fibronectin and full-length collagen IV, respectively, but had no effect on cell adhesion to RGD-α3NC1, either alone or in com-
Three wells were coated with RGD-\(\alpha3\) and \(\alpha3\)NC1 domains, and adhesion assay was performed as described under "Experimental Procedures." The data points represent the mean absorbance ± S.D. of triplicate wells. This experiment was repeated four times with similar results.

The wells were coated with RGD-\(\alpha3\) (●), RGD-\(\alpha1\) (○), or \(\alpha1\) (△) NC1 domains. The data points represent the mean absorbance ± S.D. of triplicate wells. This experiment was repeated five times with similar results.

A, HUVEC adhesion to RGD-\(\alpha3\) and \(\alpha3\)NC1 domains. The data points represent the mean absorbance ± S.D. of triplicate wells. This experiment was repeated four times with similar results.

B, HUVEC adhesion to RGD-\(\alpha3\) and RGD-\(\alpha3\)NC1 domains. The data points represent the mean absorbance ± S.D. of triplicate wells. This experiment was repeated five times with similar results.

The wells were coated with RGD-\(\alpha3\) (●), RGD-\(\alpha1\) (○), or \(\alpha1\) (△) NC1 domains. The data points represent the mean absorbance ± S.D. of triplicate wells. This experiment was repeated five times with similar results.

*Fig. 4.* Identification of HUVEC integrins bound to the RGD-\(\alpha3\)NC1 column. A, immunoprecipitation of the fraction eluted from the RGD-\(\alpha3\)NC1 column with EDTA with normal mouse IgG (lane 1) or integrin antibodies to \(\alpha\beta3\) (lane 2), \(\alpha\beta3\) (lane 3), \(\beta1\) (lane 4), and \(\alpha2\) (lane 5). Molecular masses of protein markers in kDa are indicated on the left. B, direct immunoprecipitation of biotinylated HUVEC membrane proteins with integrin antibodies to \(\alpha\beta3\) (lane 1), \(\alpha\beta5\) (lane 2), \(\alpha\beta5\) (lane 3), \(\alpha2\) (lane 4), \(\alpha5\) (lane 5), and \(\beta1\) (lane 6).

**Fig. 5.** Effect of integrin antibodies on the HUVEC adhesion to NC1 domains. A, inhibition of HUVEC adhesion by \(\alpha\beta3\) antibodies. The wells were coated with RGD-\(\alpha3\) (●), RGD-\(\alpha1\) (○), or \(\alpha1\)NC1 (△) at 10 μg/ml. Integrin \(\alpha\beta3\) neutralizing antibodies (LM-609) were preincubated with cell suspension for 30 min before adding to the wells. B, effect of \(\beta1\), \(\alpha5\), and \(\alpha\beta5\) antibodies on HUVEC adhesion to RGD-\(\alpha3\)NC1. The wells were coated with RGD-\(\alpha3\)NC1 (10 μg/ml), fibronectin (5 μg/ml), or type IV collagen from Engelbreth-Holm-Swarm tumor cells (2.5 μg/ml). HUVEC were preincubated without (lane C) or with 10 μg/ml of \(\alpha\beta5\) (BIIG2), \(\beta1\) (AIIIB2), or \(\alpha\beta5\) (P1F6) integrin blocking antibodies alone or in combination with \(\alpha\beta3\) (LM-609, 1 μg/ml). The data points/bars represent the mean absorbance ± S.D. of triplicate wells. These experiments were repeated three times with similar results.

*Fig. 5.* Contribution of the RGD and Non-RGD Motifs for Binding of Purified \(\alpha\beta3\) and \(\alpha\beta5\) Integrins to RGD-\(\alpha3\)NC1—The functionality and relative contribution of RGD and non-RGD motifs of the RGD-\(\alpha3\)NC1 domain for binding to \(\alpha\beta3\) and \(\alpha\beta5\) was determined by solid phase binding assays using purified integrins. The results show that \(\alpha\beta3\) binds to both RGD-\(\alpha3\)NC1 and \(\alpha3\)NC1 domains in a dose-dependent and saturable manner (Fig. 6A). However, the binding capacity of \(\alpha3\)NC1 is only 25% of that for the RGD-\(\alpha3\)NC1 domain, indicating a strong contribution of the RGD site. Likewise, when the RGD site is attached to the \(\alpha1\)NC1 in the RGD-\(\alpha1\)NC1 chimera, the \(\alpha\beta3\) binding is greatly increased over that of \(\alpha1\)NC1 domain (Fig. 6B). These results reveal that the RGD site is a major contributor in \(\alpha\beta3\) binding to the RGD-\(\alpha3\)NC1 domain and that the non-RGD motifs within the \(\alpha3\)NC1 domain also contribute to binding, but to a lesser extent. The functionality of the non-RGD motifs is further evident by the greater binding to the \(\alpha3\)NC1 domain over that of \(\alpha1\)NC1 as well as the RGD-\(\alpha3\)NC1 relative to the RGD-\(\alpha1\)NC1 domain.
In similar experiments with purified $\alpha_3\beta_1$ integrin, the binding to RGD-$\alpha 3 NC1$ was significantly lower compared with $\alpha_3\beta_3$ (38.5% averaged from four experiments), despite equal binding of both integrins to vitronectin. Deletion of the RGD site further decreased $\alpha_3\beta_3$ binding by 55% when compared with the RGD-$\alpha 3 NC1$ domain, indicating that the RGD motif is a binding site for both $\alpha_3\beta_2$ and $\alpha_3\beta_3$ integrins. In addition, no binding was detected of purified $\alpha_3\beta_1$, $\alpha_3\beta_3$, or $\alpha_3\beta_3$ integrins to the RGD-$\alpha 3 NC1$ domain under the same conditions (data not shown).

Contribution of the Two Non-RGD Motifs of the $\alpha 3 NC1$ Domain in Cell Adhesion and $\alpha_3\beta_3$ Integrin Binding—Utilizing short linear peptides, two RGD-independent sites within the $\alpha 3 NC1$ domain have previously been shown to promote adhesion and inhibit proliferation of endothelial and tumor cells. These sites correspond to residues 56–75 (designated peptide T3) and 185–203 of $\alpha 3 NC1$ domain (17, 18). Biological activity of both peptides was shown to be dependent on $\alpha_3\beta_3$ integrin binding. Herein, we designate these two integrin-binding sites as non-RGD motifs. Using these two peptides, we addressed whether either or both non-RGD motifs account for full cell adhesive and integrin binding activity of the whole $\alpha 3 NC1$ domain (Fig. 1A). HUVEC adhesion to the $\alpha 3 NC1$ domain was only partially (30%) inhibited by T3 peptide, whereas peptide 185–203 had no effect even at ~100-fold molar excess of soluble peptides over the immobilized $\alpha 3 NC1$ domain (Fig. 7). At the same concentration, both peptides had no effect on cell adhesion to the RGD-$\alpha 3 NC1$ domain.

The effect of T3 and 185–203 peptides on integrin binding was directly determined using solid phase assay. Binding of the $\alpha_3\beta_3$ integrin to the immobilized $\alpha 3 NC1$ domain was competitively inhibited by an excess of soluble $\alpha 3 NC1$ with an IC$_{50}$ of $\sim 0.1 \mu M$ (Fig. 8A). The inhibitory effect of T3 and 185–203 peptides was lower, with an IC$_{50}$ in the low micromolar range. The difference was even more pronounced for $\alpha_3\beta_3$ binding to the RGD-$\alpha 3 NC1$ domain (Fig. 8B), where both peptides showed only partial inhibition, consistent with the major contribution of the RGD site for binding. Simultaneous addition of both peptides did not cause further inhibition of integrin binding (data not shown). Thus, the inhibition of $\alpha_3\beta_3$ binding to both $\alpha 3 NC1$ and RGD-$\alpha 3 NC1$ domains by T3 and 185–203 peptides was at least 10-fold less potent than by whole recombinant proteins. Taken together, our data indicate that the two non-RGD peptides do not fully mimic the cell adhesive and integrin binding activity of the $\alpha 3 NC1$ domain, from which they are derived.

**RGD Peptide Inhibits Cell Adhesion and $\alpha_3\beta_3$ Integrin Binding to RGD-$\alpha 3 NC1$ and $\alpha 3 NC1$ Domain**—Given the fact that the RGD is a potent inhibitor of integrin-mediated cell adhesion to several extracellular matrix proteins, the effect of soluble RGD peptide on cell adhesion and $\alpha_3\beta_3$ integrin binding was measured. RGD peptide at 20 $\mu M$ strongly inhibited HUVEC adhesion to both $\alpha 3 NC1$ and RGD-$\alpha 3 NC1$ domains (Fig. 7). Furthermore, cell adhesion to T3 and 185–203 peptides immobilized on solid phase was also strongly inhibited by the soluble RGD peptide (data not shown).

We also used a solid phase ligand binding assay to directly assess whether soluble RGD could inhibit $\alpha_3\beta_3$ binding to the RGD-$\alpha 3 NC1$ domain. RGD peptide, at concentrations as low as 0.1 $\mu M$, completely abolished the binding of $\alpha_3\beta_3$ integrin to both RGD-$\alpha 3$ and $\alpha 3 NC1$ domains (Fig. 9), indicating that integrin binding is significantly more sensitive to the RGD peptide compared with HUVEC adhesion. The inhibition by T3 and 185–203 peptides was at least 400-fold less efficient than RGD, suggesting a higher affinity of $\alpha_3\beta_3$ integrin for RGD compared with non-RGD linear peptides. In addition, we tested...
Non-RGD peptides, previously identified as the competitive for integrin binding than the native protein, indicating the importance of additional structural and/or conformational features of the o3NC1 domain for integrin binding. Therefore, the RGD site, in addition to non-RGD motifs, may contribute to the mechanisms of endothelial cell adhesion in the human vasculature and the anti-angiogenic activity of the RGD-o3NC1 domain. This finding of a functional RGD site is contrary to a previous report (16); consequently, it impacts the understanding of the mechanism of cell adhesion and anti-angiogenic activity of the RGD-o3NC1 domain.

We demonstrate by the gain- and loss-of-function approaches that the RGD site significantly enhances the inherent capacity of the o3NC1 domain to support endothelial cell adhesion. Contrary to our findings, Maeshima et al. (16) reported that this RGD site is nonfunctional, based on the failure of a 20-mer synthetic peptide containing RGD to support adhesion and the lack of inhibition of cell adhesion to the recombinant RGD-o3NC1 domain by the cyclic RGD peptide. This discrepancy may relate to our use of recombinant proteins expressed in HEK-293 cells for native conformation, whereas the RGD-o3NC1 domain expressed in Escherichia coli has an unfolded conformation (30), and low coating efficiency or sterical constraints for short synthetic peptides immobilized on solid phase used in their studies.

The cell adhesion to both the RGD-o3NC1 and o3NC1 do-
Integrin Binding to the α3NC1 Domain of Type IV Collagen

mains is mediated by αβ3 integrin. This was initially shown for the RGD-α3NC1 domain and its deletion fragments using integrin-blocking antibodies (15, 16). In the present study, the identity of HUVEC integrins that bind the RGD-α3NC1 domain was determined by the direct approach of affinity chromatography. Among the numerous integrins expressed on endothelial cells (35), only αβ3 and αβ2 integrins bound the RGD-α3NC1 domain in a divalent cation-dependent manner. Moreover, functional studies using blocking antibodies revealed that endothelial cell adhesion to both α3NC1 and RGD-α3NC1 domain is mediated only by the αβ3 integrin.

The binding of αβ3 integrin to the RGD-α3NC1 domain involves interactions with both the proximal RGD site and non-RGD motifs within the α3NC1 domain. In solid phase binding assays, the αβ3 integrin bound to both the RGD-α3NC1 and to α3NC1 domains in a dose-dependent and saturable manner, but the RGD site enhanced the binding by 4-fold. These findings, together with results of adhesion studies, provide strong evidence that (a) αβ3 integrin mediates endothelial adhesion to RGD-α3NC1 domain through binding to both RGD and non-RGD motifs and (b) the RGD site plays a dominant role in both integrin binding and cell adhesion. The functionality of the RGD site is consistent with numerous reports on its role as a key binding motif for multiple integrins, including αβ3 (26, 36). Contrary to these findings, Maeshima et al. (16) found that the RGD site in the RGD-α3NC1 domain (domain 1) is nonfunctional for binding of αβ3 integrin on the basis of adhesion studies alone, leading them to the conclusion that endothelial adhesion is mediated exclusively by αβ3 binding to non-RGD motifs. This disparity in findings and conclusions may relate to differences in experimental strategies (see above).

The non-RGD motifs that bind αβ3 integrin were previously mapped to two sites within the α3NC1 domain, residues 56–75 and 185–203, with use of short linear peptides (17, 18). These peptides designated T3 and 185–203, supported cell adhesion of endothelial and melanoma cells, respectively. However, as shown in the present study, only T3 had a partial capacity to compete with the whole NC1 domain in cell adhesion assays, and both peptides were 10-fold less potent in competing for the binding of αβ3 integrin in solid phase binding assays. Thus, the two non-RGD peptides do not fully mimic the cell adhesion and integrin binding activities of the parental α3NC1 domain, indicating that the mechanisms of cell adhesion and integrin binding involve additional residues and/or conformational features not present in the linear peptides. It is conceivable that the non-RGD motifs, in the form of short peptides, would not adopt the same β-sheet conformation favorable for integrin binding, as they exist within the context of the native α3NC1 domain (Fig. 1, C and D). Moreover, they are located at opposite sides of the NC1 domain, suggesting the independent participation of each motif in integrin binding.

It has been proposed that the non-RGD motifs within α3NC1 domain bind to a site on αβ3 integrin distinct from the RGD-binding pocket (16, 23). This suggestion was based on the absence of the effect of RGD peptides on cell adhesion to RGD-α3NC1 domain. Contrary to this finding, however, we found that the soluble RGD peptide strongly inhibits HUVEC adhesion and integrin binding, not only to RGD-α3NC1 but also to the α3NC1 domain. Similar to our results, an inhibitory effect of RGD peptides has been reported for several other αβ3 ligands lacking the RGD sequence, such as the C-terminal fragment of MMP-2 (37), cysteine-rich heparin-binding protein Cyr-61 (38), angiostatin (39), and plasmin (40). Therefore, both the RGD and non-RGD motifs of the RGD-α3NC1 domain may bind to the identical site or spatially overlapping sites on the integrin. Alternatively, these motifs may bind to distinct pockets within the αβ3 heterodimer, which are allosterically interconnected. For example, the existence of two distinct binding pockets has been shown on α5β1 integrin for the RGD and non-RGD peptides of fibronectin (41, 42). Moreover, RGD ligands are capable of α5β1 binding even when it is already occupied by fibronectin. Thus, if similar binding sites exist within αβ3 for RGD and non-RGD motifs of RGD-α3NC1, they are likely to be mutually dependent as supported by our observation that non-RGD peptides T3 and 185–203 inhibit αβ3 binding to both α3NC1 and the RGD-dependent ligand vitronectin to a similar extent.

The α3 chain of type IV collagen is a major component of the basement membrane that underlies the endothelium of glomerular and alveolar capillaries. Our finding that αβ3 and αβ2 integrins directly interact with α3NC1 domain provides insight into the possible endogenous function of the α3 chain. For example, in the glomerular basement membrane the α3NC1 domain, as a part of the α3α4α5 network, could play a role in the attachment of endothelial cells, which express αβ3 integrin (43), contributing to glomerular integrity and ultrafiltration function. However, the accessibility of the non-RGD motifs for αβ3 integrin within the collagen IV network of basement membrane is still unknown. Homology modeling based on the crystal structure of native α1α2 NC1 hexamer (44) suggests that non-RGD integrin-binding motifs of the α3NC1 domain could be buried within the α3α4α5 hexamer and therefore not accessible for binding. It should be noted that among the known mammalian sequences, the RGD site proximal to α3NC1 domain is unique for the human species. Location of this site within the triple-helical domain of α1α2(IV) collagen, which has 11 different RGD sites, does not bind αβ integrin (45). However, phosphorylation of a serine residue immediately adjacent to RGD sequence observed in vivo indicates that the secondary structure of this region could be different from triple helix, suggesting that this RGD site may be accessible to cellular receptors (46).

Our finding that the RGD motif plays a critical role in endothelial cell adhesion strongly suggests that it contributes to the anti-angiogenic or anti-tumor activity of the RGD-α3NC1 domain. This is supported by the capacity of RGD peptides to inhibit angiogenesis and tumor growth (47–49), presumably because of their interference with the adhesion and migration of endothelial cells to extracellular matrix proteins (50). In addition, the RGD site may facilitate targeting of α3NC1 domain to tumor blood vessels, as has been shown for RGD-containing conjugates, such as doxorubicin or monoclonal antibodies (51, 52).

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