Endostatin is an endogenous inhibitor of angiogenesis. Although several endothelial cell surface molecules have been reported to interact with endostatin, its molecular mechanism of action is not fully elucidated. We used surface plasmon resonance assays to characterize interactions between endostatin, integrins, and heparin/heparan sulfate. α5β1 and αvβ3 integrins form stable complexes with immobilized endostatin ($K_D = 1.8 \times 10^{-8}$ M, two-state model). Two arginine residues (Arg27 and Arg139) are crucial for the binding of endostatin to integrins and to heparin/heparan sulfate, suggesting that endostatin would not bind simultaneously to integrins and to heparan sulfate. Experimental data and molecular modeling support endostatin binding to the headpiece of the αvβ3 integrin at the interface between the β-propeller domain of the αv subunit and the βA domain of the β3 subunit. In addition, we report that α5β1 and αvβ3 integrins bind to heparin/heparan sulfate. The ectodomain of the α5β1 integrin binds to heparin with high affinity ($K_D = 15.5$ nM). The direct binding between integrins and heparin/heparan sulfate might explain why both heparan sulfate and α5β1 integrin are required for the localization of endostatin in endothelial cell lipid rafts.

Endostatin is an endogenous inhibitor of angiogenesis that inhibits proliferation and migration of endothelial cells (1–3). This C-fragment of collagen XVIII has also been shown to inhibit 65 different tumor types and appears to down-regulate pathological angiogenesis without side effects (2). Endostatin regulates angiogenesis by complex mechanisms. It modulates embryonic vascular development by enhancing proliferation, migration, and apoptosis (4). It also has a biphasic effect on the inhibition of endothelial cell migration in vitro, and endostatin therapy reveals a U-shaped curve for antitumor activity (5, 6). Short term exposure of endothelial cells to endostatin may be proangiogenic, unlike long term exposure, which is anti-angiogenic (7). The effect of endostatin depends on its concentration and on the type of endothelial cells (8). It exerts the opposite effects on human umbilical vein endothelial cells and on endothelial cells derived from differentiated embryonic stem cells. Furthermore, two different mechanisms (heparin-dependent and heparin-independent) may exist for the anti-proliferative activity of endostatin depending on the growth factor used to induce cell proliferation (fibroblast growth factor 2 or vascular endothelial growth factor). Its anti-proliferative effect on endothelial cells stimulated by fibroblast growth factor 2 is mediated by the binding of endostatin to heparan sulfate (9), whereas endostatin inhibits vascular endothelial growth factor-induced angiogenesis independently of its ability to bind heparin and heparan sulfate (9, 10). The broad range of molecular targets of endostatin suggests that multiple signaling systems are involved in mediating its anti-angiogenic action (11), and although several endothelial cell surface molecules have been reported to interact with endostatin, its molecular mechanisms of action are not as fully elucidated as they are for other endogenous angiogenesis inhibitors (11).

Endostatin binds with relatively low affinity to several membrane proteins including α5β1 and αvβ3 integrins (12), heparan sulfate proteoglycans (glypican-1 and -4) (13), and KDR/Flk1/vascular endothelial growth factor receptor 2 (14), but no high affinity receptor(s) has been identified so far. The identification of molecular interactions established by endostatin at the cell surface is a first step toward the understanding of the mechanisms by which endostatin regulates angiogenesis. We have previously characterized the binding of endostatin to heparan sulfate chains (9). In the present study we have focused on characterizing the interactions between endostatin, α5β1, αvβ3, and αvβ5 integrins and heparan sulfate. Although interactions between several integrins and endostatin have been studied previously in solid phase assays (12) and in cell models (12, 15, 16), no molecular data are available on the binding site of endostatin to the integrins. We found that two arginine residues of endostatin (Arg27 and Arg139) participate in binding to integrins and to heparan sulfate, suggesting that endostatin is not able to bind simultaneously to these molecules displayed at
the cell surface. Furthermore, we have demonstrated that \( \alpha 5 \beta 1 \), \( \alpha v \beta 3 \), and \( \alpha v \beta 5 \) integrins bind to heparan sulfate. This may explain why both heparan sulfate and \( \alpha 5 \beta 1 \) integrins are required for the localization of endostatin in lipid rafts, in support of the model proposed by Wickström et al. (15).

**EXPERIMENTAL PROCEDURES**

*Source of Proteins and Glycosaminoglycans—* Recombinant human endostatin, the trimeric C-terminal domain of collagen XVIII called NC1 (noncollagenous 1) and several mutants (D104N and the double mutant R27A/R139A) were produced by human embryonic kidney cells expressing Epstein-Barr virus nuclear antigen (293-EBNA cells) according to established protocols (9, 10). Amino acids residues were numbered starting from the first amino acid residue of endostatin (His1, also referred to as His\(^{1,2,3}\) when numbering starts from the first amino acid of the entire C-terminal domain NC1 of collagen XVIII). Wild type and mutant proteins were tagged by the pepti
dipeptide FLAG at the N-terminal end for endostatin and at the C-terminal end for the NC1 domain. The conditioned culture media were filtered through 0.22-

![micron filters](image-url) and applied to an anti-FLAG M2 column (3.5 ml; Sigma) equilibrated in 0.01 M phosphate-buffered saline, pH 7.4, containing 0.138 M NaCl, 0.0027 M KCl, and 0.05% Tween 20 (P3563; Sigma). The recombinant proteins were eluted by soluble FLAG peptide (150 \( \mu g/ml \)). Endostatin and the NC1 domain were further purified by gel filtration on a Superdex 75 column (2.6 \( \times \) 60 cm; GE Healthcare) and on a Sephacryl S200 column (2.6 \( \times \) 60 cm; GE Healthcare), respectively. The purity of recombinant proteins was assessed by SDS-PAGE and by Western blot. The purified proteins were concentrated by ultrafiltration and stored at –80°C. Chondroitin sulfate from bovine trachea (C8529), heparin (HP) (H3393), and dermatan sulfate (C3788) from porcine intestinal mucosa, human fibronectin (F2006), GRGDSPK peptide (G4144), and GRGDSPK (G1269) were purchased from Sigma-Aldrich. Heparan sulfate from porcine intestinal mucosa, human fibronectin (F2006), GRADSPK peptide (Sigma), and GRGDSPK (Sigma) was used as a control. Inhibition assays were also performed with an endostatin peptide at 50 and 150 \( \mu g/ml \) (ES-2) (16).

The binding of \( \alpha 5 \beta 1 \), \( \alpha v \beta 3 \), and \( \alpha v \beta 5 \) integrins to heparin/ heparan sulfate was investigated using biotinylated heparin/ heparan sulfate captured onto a streptavidin sensor chip as previously described (9). An immobilization level ranging between 70 and 200 resonance units was obtained. A control flow cell was prepared by immobilizing only streptavidin. Full-length \( \alpha 5 \beta 1 \), \( \alpha v \beta 3 \), and \( \alpha v \beta 5 \) integrins (30 \( \mu g/ml \)) were injected at 20 \( \mu l/min \) for 4 min over immobilized glycosaminoglycans with 25 \( \mu M \) Tris, pH 7.5, containing 150 mM NaCl, 0.1 mM CaCl\(_2\), 2 mM MgCl\(_2\), and 0.005% P20 as running buffer. Kinetic parameters were calculated from a set of sensorgrams collected by injection over immobilized heparin and heparan sulfate of several concentrations of \( \alpha 5 \beta 1 \) integrin (6–160 and 6–200 \( nM \), respectively). Some experiments were carried out in the presence of several cation chelators (10 mM BAPTA, \(^3\) 10 mM EDTA, and 10 mM EGTA) to determine the influence of divalent cations on integrin binding. Inhibition experiments were performed by preincubating several glycosaminoglycans (heparin, heparan, dermatan, and chondroitin sulfate) at 5 \( \mu g/ml \) with integrins (30 \( \mu g/ml \)) for 1 h at room temperature before injection over immobilized heparin/heparan sulfate. Another set of inhibition experiments was carried out with heparin and heparan sulfate oligosaccharides of defined size (5 \( \mu g/ml \), generous gift from Dr. Rabia Sadir, Institut de Biologie Structurale, UMR 5075, Grenoble, France) and the full-length and truncated ectodomains of \( \alpha 5 \beta 1 \) integrin (40 \( \mu g/ml \)).

The full-length (\( \alpha 5E951 \beta 1D708 \), 2–78 \( nM \)) and truncated (\( \alpha 5D613 \beta 1P455 \), 20–480 \( nM \)) ectodomains of \( \alpha 5 \beta 1 \) integrin were injected over immobilized heparin at 20 \( \mu l/min \) for 4 min to calculate kinetics and affinity parameters. The running buffer was 25 mM Tris, pH 7.4, containing 150 mM NaCl, 0.005% P20, 1 mM MnCl\(_2\), and 2 mM MgCl\(_2\).

To check whether some interactions took place in a cellular context, we injected three Chinese hamster ovary (CHO) cell

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\(^3\) The abbreviations used are: BAPTA, 1,2-bis(2-aminophenoxyethyl)-

-N,N’,N’-tetraacetic acid; ABTS, 2,2’-azino-bis(3-ethylbenzothiazoline-6- sulfonic acid; CHO, Chinese hamster ovary; ES, endostatin, SPR, surface plasmon resonance.
lines over immobilized integrins or heparin/heparan sulfate. We used the CHO-K1 cell line (ATCC CCL-61), one mutant CHO cell line defective in glycosaminoglycan biosynthesis (CHO-745 cells ATCC: CRL-2242 (18), and the CHO B2 cell line lacking the α5 subunit (19), a generous gift from Dr. R. Juliano (Chapel Hill, NC). The cells were harvested by treatment with 1% EDTA at ~80–90% confluence and were suspended (3 × 10^5 cells/ml) in 25 mM Tris, pH 7.4, containing 150 mM NaCl, 1 mM MgCl2, 0.1 mM CaCl2, and P2O 0.005%. They were stored in a water bath at 37 °C without shaking until injection and were examined under an optical microscope to check cell viability before injection at 30 μl/min for 5 min over immobilized heparin or heparan sulfate in a Biacore 3000. The sensor chip surface was regenerated with a pulse (30–60 s) of 0.025–0.033% SDS and 1.5–2 μM NaCl. This experimental design did not mimic cell adhesion because the cells were injected in a buffer flow. Control sensograms were automatically subtracted from the sensograms obtained with immobilized proteins or glycosaminoglycans to yield true binding responses.

Solid Phase Ligand Binding Assays—Soluble full-length α5β1, αvβ3, and αvβ5 integrins (4, 8, and 16 μg/ml) were coated into wells of a 96-well microplate (Immulon 4HBX) in phosphate-buffered saline overnight at room temperature. Casein was used as a negative control. The coating solution (100 μl) was removed and replaced with 5% bovine serum albumin in Tris-buffered saline (25 mM Tris, pH 7.4, 0.1% bovine serum albumin, 1 mM MnCl2) and incubated for 3 h at room temperature. Biotinylated heparin was used as a ligand, diluted to the required concentration (25 μg/ml) in Tris-buffered saline, and incubated for 3 h at 37 °C. The plate was washed three times with Tris-buffered saline before addition of extravidin-peroxidase (1:500 dilution in Tris-buffered saline overnight at room temperature. 100 μl of 1% (v/v) ABTS in 0.1 M sodium acetate, 0.05M NaH2PO4, pH 5.0, 0.01% H2O2 (v/v) was added. Absorbance readings were measured at 405 nm on a Dynex MRX 96-well microplate reader. Absorbance reading was taken 30 min after addition of the reagent. Each sample was assayed in triplicate, and binding to casein was subtracted from all measurements.

Molecular Modeling of Endostatin-Integrin Interactions—Docking experiments were performed using the ZDOCK program (20). The following structures were extracted from the Protein Data Bank: endostatin (1BNL) and the extracellular domain of the αvβ3 integrin either in the free (1JV2) or the RGD-bound form (1L5G). Because of the limited size of protein structure handled by the ZDOCK program, only part of the extracellular domain of the αvβ3 integrin was used for docking experiments. This included the β-propeller and thigh domains of the αv subunit (Phe1–Leu 594), and the βA hybrid and plexin-semaphorin-integrin domains of the β3 subunit (Glu59–Asp 634) were used. The integrin was kept fixed, and endostatin was rotated and translated using a 6° sampling density in the rotational space. The top ZDOCK predictions were reranked with an optimized energy function using ZRANK (21). Minimization was run with Sybyl® software (Tripos) using the Amber 7 FF99 force field and Powell minimization method (gradient 0.5 Å, 500 iterations). The refined conformations were used to identify the amino acids participating in the interaction. Energetically important amino acid residues in endostatin-integrin interface were predicted using computational alanine scanning (22). The input consists of a three-dimensional structure of a protein-protein complex; output is a list of “hot spots” or amino acid side chains that are predicted to significantly destabilize the interface when mutated to alanine, analogous to the results of experimental alanine-scanning mutagenesis (23).

Cell Adhesion Colorimetric Assays and Cell Morphology Analysis—Chinese hamster ovary wild type cells CHO-K1 (ATCC CCL-61), CHO-745 (ATCC CRL-2242), and CHO-677 (ATCC CRL-2244) cell lines defective in glycosaminoglycans were used for cell adhesion assays. CHO-745 cells have less than 6% of the amount of sulfated glycosaminoglycans present on the wild type cell line, and the CHO-677 cell line has 50% of cell sulfated glycosaminoglycan with decreased heparan sulfate but increased chondroitin sulfate compared with the wild type cell line (18). CHO-K1, CHO-677, and CHO-745 were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, nonessential amino acids, and gentamycin (Sigma-Aldrich). Prior to adhesion assays, the cells were harvested with 1% EDTA in phosphate-buffered saline and suspended in serum free Dulbecco’s modified Eagle’s medium. Colorimetric cell adhesion assays were performed as previously described (24). 96-Well plates (Costar, France) were coated overnight at 4 °C with α5β1 integrin (Chemicon, CC1027) at a concentration range of 0–40 μg/ml (0.1 μl/well). Fibronectin was used as a positive control for cell adhesion assays. After saturation with 1% bovine serum albumin, freshly suspended cells in serum-free Dulbecco’s modified Eagle’s medium (5 × 10^5 cells/ml) were plated onto wells (0.1 μl/well) and allowed to attach at 37 °C for 40 min. Then the nonadherent cells were gently rinsed with phosphate-buffered saline, fixed with 1% glutaraldehyde, and stained with 0.1% crystal violet in water. After extensive washing, the dye fixed to the cells was solubilized with 0.2% Triton X-100, and the absorbance was read at 570 nm in an enzyme-linked immunosorbent assay plate reader (Dynex MRX). Each assay was performed in triplicate. The morphology of the adherent cells was observed with a Nikon TE300 microscope. The images were collected and processed digitally with Photoshop 8.0 (Adobe). Cell morphology was analyzed with Image J software on 100 cells (CHO-K1, CHO-677, and CHO-745; n = 4), and the circularity index was measured. As the circularity index approaches 0, it indicates an increasingly elongated shape, whereas a value of 1 indicates a perfect circle.

RESULTS

α5β1 and αvβ3 Integrins form Stable Complexes with Immobilized Endostatin—Endostatin has been reported to bind to purified α5β1 integrin in a specific manner in a solid phase binding assay (12). In SPR binding assays, endostatin bound to immobilized α5β1 and αvβ3 integrins (Fig. 1, A and B) and to αvβ5 integrin (data not shown). Fibronectin, used as a positive control, bound to α5β1 and αvβ3 integrins under the same experimental conditions (Fig. 1C). The association and dissociation rates were very fast, indicating that the binding of endostatin to immobilized integrins was transient. The sensograms were best fitted to a heterogeneous ligand model, sug-
**Endostatin, Integrins, and Heparan Sulfate**

**FIGURE 1.** SPR binding assays. Injection of soluble endostatin (119–1904 nM) over α5β1 (A) and αvβ3 (B) integrins immobilized on a sensor chip. Injection of fibronectin (23 nM, C) over α5β1 (dashed line) and αvβ3 integrin (solid line), immobilized on the sensor chip. Injection of soluble α5β1 (15–240 nM, D) and αvβ3 (12.5–200 nM, E) integrins over endostatin immobilized on a sensor chip (flow rate, 30 μl/min; contact time, 180 s; CM3 sensor chip) are shown.

suggesting that endostatin might bind to two independent sites on immobilized integrins. This might be due to a possible heterogeneity in integrins immobilized on the sensor chip surface via distinct lysine residues and/or to the fact that the integrin preparations containing a mixture of conformers. Affinity, but not kinetic, constants governing the binding of endostatin to immobilized α5β1 or to αvβ3 integrins were similar. The two equilibrium affinity constants were $12.0 \times 10^{-7} \text{ versus } 9.75 \times 10^{-7}$ M for one site and $5.01 \times 10^{-7} \text{ versus } 4.51 \times 10^{-7}$ M for the other (Table 1). The affinity constant ratio was 1.23 and 1.11, respectively, the highest $K_D$ values being obtained for the αvβ3 integrin.

In contrast, the complexes formed by immobilized endostatin with soluble α5β1 and αvβ3 integrins were much more stable (Fig. 1, D and E). The affinity, calculated with a two-state reaction model, is higher when endostatin is immobilized (Table 1).

**D104N Endostatin Mutant Retains the Ability to Interact with α5β1 and αvβ3 Integrins**—The binding properties of the D104N mutant were investigated because individuals homozygous for the D104N polymorphism in the COL18A1 gene have a high risk of occurrence of sporadic breast cancer (25). In addition, this polymorphism may influence the age of onset of acute myeloid leukemia (26). The D104N mutation did not alter endostatin folding as assessed by fluorescence and circular dichroism spectra (data not shown). The D104N mutant retained its ability to bind to α5β1 and αvβ3 integrins in SPR binding assays (data not shown). The best interaction model was the two-state model in both orientations (soluble or immobilized D104N mutant) (Table 1). The apparent affinity was higher when the D104N mutant is immobilized (Table 1). The affinity was similar to that calculated with wild type endostatin, although the D104N mutant bound to immobilized α5β1 and αvβ3 integrins to a greater extent than the wild type endostatin. The binding level was increased by 201% for α5β1 integrin and 188% for αvβ3 integrin. These results show that the D104N mutation does not affect the binding affinity of endostatin to integrins, although it does affect the binding level.

Arg27 and Arg139 Participate in the Binding of the NC1(XVIII) Domain to Integrins—Endostatin is released in vivo as a monomeric protein. Proteolytic release of the C-terminal trimeric domain of collagen XVIII, NC1(XVIII), containing three endostatin monomers (Fig. 2A), also occurs in cell cultures and tissues (27), and we investigated its ability to bind integrins. The NC1(XVIII) bound to immobilized α5β1 and αvβ3 integrins (Fig. 2, B and C). The dissociation rates were lower than those observed for monomeric endostatin, suggesting that the trimerization and/or the N-terminal part of the NC1 domain increases the stability of the interaction. The binding was also observed in the reverse orientation when soluble α5β1 and αvβ3 integrins were injected over immobilized NC1 domain (data not shown).

The D104N mutant of the NC1 domain bound to immobilized α5β1 and αvβ3 integrins to a greater extent than the wild type NC1, the binding level being increased by 149 and 151%, respectively (Fig. 2, A and B). Because the double R27A/R139A monomeric endostatin mutant has lost its capacity to bind heparin (28), it was interesting to determine whether these two arginine residues are also involved in the binding to other endostatin or NC1(XVIII) partners. The double mutation R27A/R139A of the NC1 domain strongly decreased the bind-
Kinetic and affinity parameters governing the binding of wild-type (Wt) or mutant ES and of the C-terminal NC1 domain of collagen XVIII to α5β1 and αvβ3 integrins. Rate constants and affinity constant were calculated in both orientations (i.e. endostatin in solution, analyte, or immobilized, ligand) using the Biacore 4.1 software. \( k_a \) and \( k_d \) association rate constants; \( k_{diss} \) dissociation rate constants characterizing the two binding sites in the heterogeneous ligand model used to fit the experimental data, or the formation of the two complexes in the two-state reaction model (BIAeval 4.1 software).

| Ligand       | Analyte | Model                  | \( \chi^2 \) | \( k_a \)       | \( s^{-1} \) | \( k_{diss} \) | \( k_{diss} \) | \( K_D \) | \( s^{-1} \) |
|--------------|---------|------------------------|-------------|-----------------|-------------|-----------------|-----------------|-------------|-------------|
| α5β1         | Wt ES   | Heterogeneous ligand   | 0.793       | 1.18 \( \times 10^{-4} \) | 1.15 \( \times 10^{-2} \) | 1.55 \( \times 10^{-5} \) | 6.99 \( \times 10^{-2} \) | 9.75 \( \times 10^{-2} \) | 4.51 \( \times 10^{-7} \) |
| αvβ3         | Wt ES   | Heterogeneous ligand   | 2.89        | 1.35 \( \times 10^{-5} \) | 6.77 \( \times 10^{-2} \) | 6.82 \( \times 10^{-3} \) | 8.19 \( \times 10^{-2} \) | 12.0 \( \times 10^{-2} \) | 5.01 \( \times 10^{-7} \) |
| α5β1         | D104N mutant | Two-state reaction | 1.38        | 9.51 \( \times 10^{-2} \) | 5.75 \( \times 10^{-2} \) | 3.22 \( \times 10^{-3} \) | 4.47 \( \times 10^{-2} \) | 3.51 \( \times 10^{-5} \) |
| αvβ3         | D104N mutant | Two-state reaction | 3.29        | 4.99 \( \times 10^{-3} \) | 4.48 \( \times 10^{-2} \) | 2.03 \( \times 10^{-2} \) | 2.98 \( \times 10^{-2} \) | 5.35 \( \times 10^{-6} \) |
| Wt ES        | α5β1    | Two-state reaction     | 0.607       | 1.00 \( \times 10^{-5} \) | 1.70 \( \times 10^{-2} \) | 1.42 \( \times 10^{-2} \) | 1.72 \( \times 10^{-2} \) | 1.83 \( \times 10^{-8} \) |
| Wt ES        | αvβ3    | Two-state reaction     | 0.807       | 1.06 \( \times 10^{-5} \) | 1.71 \( \times 10^{-2} \) | 1.26 \( \times 10^{-2} \) | 1.54 \( \times 10^{-2} \) | 1.75 \( \times 10^{-8} \) |
| D104N mutant | α5β1    | Two-state reaction     | 0.526       | 6.95 \( \times 10^{-4} \) | 1.14 \( \times 10^{-2} \) | 1.05 \( \times 10^{-2} \) | 1.60 \( \times 10^{-2} \) | 2.17 \( \times 10^{-5} \) |
| D104N mutant | αvβ3    | Two-state reaction     | 0.517       | 8.18 \( \times 10^{-4} \) | 1.38 \( \times 10^{-2} \) | 1.06 \( \times 10^{-2} \) | 1.57 \( \times 10^{-2} \) | 2.18 \( \times 10^{-5} \) |

* Unit of measure, \( M^{-1} s^{-2} \).

Endostatin, Integrins, and Heparan Sulfate

FIGURE 2. Binding of the trimeric NC1(XVIII) domain to integrins. A, organization of the NC1 domain of collagen XVIII, containing endostatin. Binding of wild type NC1 domain (solid line), D104N NC1 mutant (short dashed line), and R27A/R139A NC1 mutant (long dashed line) (19 nM) over immobilized α5β1 integrin (B) and αvβ3 integrin (C) (flow rate, 30 μl/min; contact time, 180 s).

Molecular Modeling of Endostatin-Integrin Interactions—

The crystal structure of the extracellular domain of the human αvβ3 integrin (29) was used for molecular modeling. Molecular modeling was performed using the original crystal structure of integrin αvβ3, which revealed a bent conformation of the head region associated with low affinity for ligand, but the bent form of αvβ3 can still bind to fibronectin (30). The predicted models were reranked using ZRANK and examined, taking into account the fact that the D104N endostatin mutant retains its ability to bind to integrins, whereas the double R27A/R139A mutant abolished it. We selected two models in which at least one of these two arginine residues was in contact with the extracellular domain of the αv and/or the β3 subunit. In both models, endostatin bound to the headpiece of the αvβ3 integrin at the interface between the β-propeller domain of the αv subunit and the β3 domain of the β3 subunit (Fig. 3, A and B). The binding site involved a basic cluster on endostatin (Fig. 3, C and D) and an acidic cluster on the integrin (Fig. 3, E and F). In the first model the amino acid residues that seemed to be particularly involved through hydrogen bonding were Arg22, Gly44, Arg53, Arg63, Arg66, Arg20, Thr131, Glu132, Glu136, Arg139, and Thr140 on endostatin; Lys125, Asp126, Asp179, Thr182, Arg214, Glu230, Asp251, Glu312, and Asn313 on the β3 integrin subunit; and Ile147, Asp148, Asp150, and Gln214 on the αv integrin subunit. In the second model, the amino acid residues that seemed to be particularly involved through hydrogen bonding were Gly22, Arg22, Arg27, Gln35, Arg38, Ala39, Arg53, Arg139, Thr140, Glu141, and Asn176 on endostatin; Tyr122, Tyr166, Asp179, Thr182, Arg214, Asn215, Gln220, Asp251, and Asn313 on the β3 integrin subunit; and Ile147, Asp148, Asp150, Tyr178, Gln214, and Asp219 on the αv integrin subunit.

We used computational alanine scanning to study the interaction free energies in the endostatin-integrin complex. Alamine scanning was performed by computationally performing the mutation of each residue sequentially and evaluating the difference in energy. This allows from a single simulation an estimate of the individual contribution of each residue to the binding. The results provide information about the positive or negative energetic consequences of mutating each residue in the sequence (22, 23). A positive value (>1 kcal/mol) of ΔΔG indicates that the mutation is predicted to destabilize the complex, whereas a negative value indicates a stabilizing effect. In the first model, computational alanine scanning of Arg22, Phe34, Thr35, Arg47, Arg53, Leu54, Arg63, Arg139, Arg139, and Asn176 in endostatin were predicted to destabilize the endostatin-integrin complex (supplemental Table S1). The residues predicted to be hot spots on the αv subunit were Tyr122, Gln214,
and Ile<sub>216</sub>, whereas Ser<sub>121</sub>, Tyr<sub>122</sub>, Ser<sub>123</sub>, Asp<sub>179</sub>, Arg<sub>214</sub>, Asp<sub>251</sub>, and Met<sub>335</sub> were predicted for the α<sub>5</sub>β<sub>3</sub> subunit. In the second model, computational mutation of four arginine residues (Arg<sub>24</sub>, Arg<sub>27</sub>, Arg<sub>47</sub>, and Arg<sub>53</sub>) of Asp<sub>30</sub>, Phe<sub>31</sub>, Phe<sub>34</sub>, and Gln<sub>35</sub> were predicted to destabilize the endostatin-integrin complex, Arg<sub>24</sub> being the “hottest” spot on endostatin. Regarding the integrin, the residues predicted to be hot spots are Tyr<sub>178</sub>, Gln<sub>214</sub>, and Ile<sub>216</sub> on the αv subunit, and Tyr<sub>122</sub>, Asn<sub>215</sub>, and Glu<sub>220</sub> (the hottest spot on integrin) on the β<sub>3</sub> subunit (supplemental Table S2).

Endostatin was predicted to bind at, or near, the ligand-binding site on the α<sub>v</sub>β<sub>3</sub> integrin. α<sub>v</sub>β<sub>3</sub> is a RGD-recognizing integrin, and docking experiments were also performed with the RGD-bound form of the α<sub>v</sub>β<sub>3</sub> integrin, but we failed to predict the same models of the integrin-endostatin complex using the RGD-bound form of the integrin. This result confirmed the results from the docking experiments performed with the RGD-free form of the integrin and prompted us to perform SPR inhibition assays to determine whether the RGD peptide could compete with endostatin for integrin binding.

**SPR Inhibition Assays**—α<sub>5</sub>β<sub>1</sub> and α<sub>v</sub>β<sub>3</sub> integrins were injected with or without preincubation with a RGD-containing peptide over immobilized endostatin. The linear peptide GRGDSPK inhibited the binding of integrins to endostatin by 80.1 and 98%, respectively, compared with the control peptide GRADSPK, which did not significantly inhibit the interaction (Fig. 4, A and B). This suggests that endostatin binds to the same site as the RGD peptide on α<sub>v</sub>β<sub>3</sub> integrin, and it supports the model of the endostatin-integrin complex predicted by molecular modeling.

α<sub>5</sub>β<sub>1</sub> and α<sub>v</sub>β<sub>3</sub> integrins were also preincubated with a peptide corresponding to amino acid residues 60–70 of endostatin and containing three surface exposed arginine residues (Arg<sup>62</sup>, Arg<sup>63</sup>, and Arg<sup>66</sup>). This peptide, termed ES-2, is able to promote endothelial cell adhesion and spreading via heparin- and integrin β<sub>1</sub>-dependent interactions, and these arginine residues...
Two Endostatin Partners, Integrins, and Heparan Sulfate Bind to Each Other in Vitro—Integrins are receptors for extracellular matrix proteins. Heparan sulfate chains are displayed at the cell surface, mostly as part of two proteoglycan families, syndecans and glypicans. Although it seemed unlikely that endostatin could bind simultaneously to integrins and to heparan sulfate because Arg$^{27}$ and Arg$^{130}$ residues participate in the binding of endostatin to both integrins and heparan sulfate, we tried to determine whether heparin was able to inhibit the binding of integrins to endostatin. Interestingly, it turned out by SPR binding assays that $\alpha$5$\beta$1 (Fig. 5A), $\alpha$v$\beta$3, and $\alpha$v$\beta$5 integrins (data not shown) also bound to immobilized heparin or heparan sulfate by SPR binding assays. Kinetic and affinity parameters were calculated from a set of sensorgrams collected by injection of several concentrations of $\alpha$5$\beta$1 integrin over immobilized heparin/heparan sulfate (Fig. 5B). The experimental data were best fitted to a two-state model involving a conformational change in the first complex formed between the integrin and heparin or heparan sulfate leading to the formation of a second, more stable complex. The rate constants were $k_{a1} = 740 \text{ M}^{-1}\text{s}^{-1}$, $k_{d1} = 0.0196 \text{s}^{-1}$, $k_{a2} = 5.52 \times 10^{-3} \text{s}^{-1}$, and $k_{d2} = 2.51 \times 10^{-8} \text{s}^{-1}$ ($R^2 = 1.23$) for the $\alpha$5$\beta$1-heparin complex and $k_{a1} = 751 \text{ M}^{-1}\text{s}^{-1}$, $k_{d1} = 0.0126 \text{s}^{-1}$, $k_{a2} = 3.94 \times 10^{-3} \text{s}^{-1}$, and $k_{d2} = 5.43 \times 10^{-4} \text{s}^{-1}$ ($R^2 = 0.422$) for the $\alpha$5$\beta$1-heparan sulfate complex. The apparent affinity constants calculated using the BIAeval software were in the micromolar range ($K = 1.14 \mu\text{M}$ for heparin and $K = 2.04 \mu\text{M}$ for heparan sulfate). The binding of full-length $\alpha$5$\beta$1, $\alpha$v$\beta$3, and $\alpha$v$\beta$5 integrins to heparin was confirmed by solid phase assays, which showed that heparin bound to a greater extent to $\alpha$v$\beta$5 integrin than to $\alpha$5$\beta$1 and $\alpha$v$\beta$3 integrins (Fig. 5C).

We focused on the binding of $\alpha$5$\beta$1 integrin to heparin because soluble $\alpha$5$\beta$1 integrin binds to immobilized endostatin in solid phase assays and because human endothelial cell adhesion on immobilized endostatin has been reported to be primarily mediated by the $\alpha$5$\beta$1 integrin (12). The specificity of...
integrin binding to heparin/heparan sulfate was checked by SPR inhibition experiments with several glycosaminoglycans. The binding of α5β1 integrin to heparin or heparan sulfate was abolished by heparin and strongly inhibited (74.8 and 77.8%, respectively) by heparan sulfate. Dermatan sulfate was a far less potent inhibitor of the binding of α5β1 integrin to heparin and heparan sulfate (30 and 50.3%, respectively), whereas chondroitin sulfate had no inhibitory activity at all (data not shown).

We determined the minimal size of heparin required to promote efficient binding of the α5β1 integrin by performing inhibition experiments with heparin oligosaccharides of various sizes (degree of polymerization). The inhibition of α5β1 integrin binding to heparin and heparan sulfate increased with the length of oligosaccharides and then reached a plateau. An octasaccharide of heparin was found to be sufficient to inhibit the binding of α5β1 integrin to heparin and heparan sulfate by more than 50% (Fig. 5D).

We also investigated the role of cations in heparin binding to α5β1 integrin. The addition of 10 mM EDTA inhibited the binding of α5β1 integrin to heparin by 47% and to heparan sulfate by 61.4%. 10 mM EGTA inhibited the binding of α5β1 integrin to heparin by 49.1% and to heparan sulfate by 61.9%. BAPTA, which is a highly selective calcium chelating reagent, abolished the binding of α5β1 integrin to the two glycosaminoglycans, the binding to heparin was abolished, and the binding to heparan sulfate was inhibited by 95%. The binding of α5β1 integrin to heparin and heparan sulfate is thus calcium-dependent.

In SPR inhibition experiments, a RGD peptide inhibited the binding of α5β1 integrin to heparin by 23.3% and to heparan sulfate by 32.6%, suggesting that binding sites of RGD and heparin/heparan sulfate on the α5β1 integrin might only partially overlap. It is unlikely that heparin/heparan sulfate and endostatin could bind to the same site on the extracellular domain of integrins because endostatin is predicted to bind to a negatively charged area on αβ3 integrin as described above, and this would be repulsive for heparin and heparan sulfate because of their strong polyanionic character.

**Heparin/Heparan Sulfate Bind to the Extracellular Domain of α5β1 Integrin**—We looked for the presence of consensus sequences BBXB or BBBXXB for heparin binding in the primary sequences of integrin subunits. As shown in Table 2, α5β1 and αβ5 integrins contain a consensus sequence for heparin binding in their extracellular domain. However, the αβ3 integrin, which lacks such a consensus sequence, was able to bind heparin, suggesting that heparin-binding sites on integrins cannot be accurately predicted by those consensus sequences. It is thus likely that amino acid residues contributing to the binding site are close to each other in the three-dimensional structure of integrins but not in their primary sequence. Although they were not useful for mapping heparin-binding sites on integrins, these consensus sequences raised a concern because the αβ subunit contains one consensus sequence for heparin binding in its intracellular domain. This prompted us to perform binding assays with soluble integrin ectodomains to confirm that heparin/heparan sulfate bound to the extracellular domain of integrins. Among the integrins investigated in this study, only the ectodomain of α5β1 integrin was available to us.

SPR assays performed with the ectodomain of the α5β1 integrin confirmed that it tightly bound to heparin and heparan sulfate. The dissociation rate was very slow, and the complexes formed between the ectodomain of the integrin and heparin were more stable than those formed with the full-length integrins. The truncated ectodomain corresponding to the minimal α5β1 Fc integrin (17) bound heparin and heparan sulfate (Fig. 6, A and B) to a lower extent than full-length α5β1 integrin but to a greater extent than the full-length α5β1 ectodomain. The difference between the full-length and the truncated ectodomains of the integrins was emphasized in Fig. 6 (A and B), where the two domains have been injected.
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Do Integrins Bind to Heparin/Heparan Sulfate at the Cellular Level? We wanted to check whether heparan sulfate chains were able to bind integrins in a cellular context and specifically whether heparan sulfate chains located at the cell surface were able to bind to integrins. We selected CHO cells (K1) for this purpose because they express high levels of endogenous α5β1 integrin and because several mutant cell lines lacking either the α5 integrin subunit (CHO-B2) or sulfated glycosaminoglycans at their cell surface (CHO-745) were available. The use of cells in SPR binding assays have been previously reported (31–33).

When wild type CHO-K1 cells were injected in buffer flow over immobilized heparin, they formed very stable complexes with heparin and heparan sulfate. Mutant CHO cells lacking both heparan and chondroitin sulfate (CHO-745) on their surface bound to immobilized glycosaminoglycans to the same extent than wild type cells (Fig. 7A). The total sulfated glycosaminoglycan present on the cell surface of the 745 cell line is less than 6% of the amount present on the wild type cell line (18). In contrast, CHO cells lacking the α5 subunit bound to a lower extent to heparin/heparan sulfate. The residual binding might be due to the presence of other integrins than α5β1 at the surface of the CHO cells. Indeed it has been shown that wild type CHO cells express high levels of endogenous αv integrins (34), which are also able to bind heparin/heparan sulfate. We performed inhibition experiments to assess the specificity of cell binding. The binding to heparin was strongly inhibited by heparin (81 and 96% inhibition for K1 and B2 cells, respectively) and to a lesser extent by chondroitin sulfate (39 and 23% for K1 and B2 cells, respectively), suggesting that the binding could be indeed mostly mediated by heparan sulfate in vivo. The binding was inhibited by 40% in the presence of 20 mM EDTA, suggesting that divalent cations participate in the binding.

CHO-K1 cells also bound to immobilized α5β1 integrin in SPR experiments (data not shown). The specificity of the binding was checked by SPR inhibition assays. Heparin inhibited the binding of the truncated α5β1 ectodomain to immobilized heparin (100% inhibition) and partially inhibited (26%) by chondroitin sulfate and heparan sulfate (20%), whereas carboxymethyl dextran did not inhibit significantly the interaction (10%). The truncated ectodomain binds to heparin with a higher affinity ($K_D = 2.09$ nM, $k_a = 1.3 \times 10^4$ M$^{-1}$ s$^{-1}$, $k_d = 2.7 \times 10^{-5}$ s$^{-1}$, and $\chi^2 = 0.0797$) than the full-length ectodomain ($K_D = 15.5$ nM, $k_a = 1.47 \times 10^4$ M$^{-1}$ s$^{-1}$, $k_d = 2.28 \times 10^{-5}$ s$^{-1}$, and $\chi^2 = 0.0813$). Both interactions were best described by a 1:1 binding model.

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FIGURE 7. A, SPR binding assays. Wild type (solid line), CHO-B2 cells (short dashed line), and CHO-745 cells (long dashed line) injected in buffer flow over immobilized heparin (flow rate, 30 µl/min; contact time, 300 s). B, cell adhesion assays. Dose-response curves of CHO-K1 (○) and CHO-745 (▲) cells to α5β1 integrin. C, dose-response curves of CHO-K1 (○) and CHO-677 (■) cells to α5β1 integrin. D, effect of glycosaminoglycan deficiencies on cell spreading on α5β1 integrin; light micrographs of cells after attachment to α5β1 integrin for 40 min. E, circularity index of α5β1-adherent CHO-K1 (gray bars), CHO-677 (white bars), and CHO-745 (black bars) cells.

over immobilized glycosaminoglycans at the same concentration (100 nM) as the full-length integrin. To show that reliable SPR signals were obtained for the two ectodomain constructs, different concentrations of the full-length (20–480 nM) and of the truncated ectodomains (2–78 nM) were injected over immobilized heparin in another set of experiments (Fig. 6, C and D). In these experimental conditions, the binding level reached ~200 resonance units, showing unambiguously that both constructs are able to bind to heparin.

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CHO-K1 cells also bound to immobilized α5β1 integrin in SPR experiments (data not shown). The specificity of the binding was checked by SPR inhibition assays. Heparin inhibited the
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binding by 68%, and chondroitin sulfate by 27%, whereas dermatan sulfate had a very limited inhibitory activity (14%). Cell adhesion assays were performed to support SPR assays. Fibronectin was used as a control for cell experiments (supplemental Fig. S3). When plated on α5β1 integrin, wild type CHO-K1 cells were able to adhere and to spread on it, whereas mutant CHO-677 and CHO-745 cell lines defective in sulfated glycosaminoglycans to various degrees retained their capacity to adhere on α5β1 integrin (Fig. 7, B and C) but spread to a lesser extent than wild type CHO-K1 (Fig. 7D). This was confirmed by morphology analysis. Indeed, a greater number of CHO cells with glycosaminoglycan deficiencies (42.86% for CHO cells with glycosaminoglycan deficiencies) had the highest circularity index greater than 0.851, whereas only 31% of the CHO-K1 cells fell into this category (Fig. 7E). CHO-745 cells, lacking heparan sulfate and having less than 6% of the amount of sulfated glycosaminoglycans present on the wild type cells (18) had the highest circularity index. Our data show that surface glycosaminoglycans are required for the cells to spread on the α5β1 integrin and that the interactions between this integrin and heparan sulfate participate in the spreading process.

DISCUSSION

We characterized in this study the binding of endostatin to α5β1 and αvβ3 integrins using SPR binding assays. The kinetics of the interaction differed markedly when endostatin was immobilized on the sensor chip or injected in a soluble form over immobilized integrins. This different behavior might contribute to the different roles previously reported for these two forms of endostatin. Indeed, immobilized endostatin promotes integrin-dependent endothelial cell migration and survival, whereas soluble endostatin inhibits integrin-dependent endothelial cell migration and survival (12) and behaves as an inhibitor of angiogenesis. This might be related to the in vivo situation, where endostatin is either immobilized within basement membranes when it is part of collagen XVIII (35) or under a soluble form in plasma (27).

Although interactions between several integrins and endostatin have been studied previously in solid phase assays (12) and in cell models (12, 15, 16), no molecular data are available on the binding site of endostatin to the integrins. We have identified the headpiece of the αvβ3 integrin as the endostatin-binding site, and we propose two models of the binary complex formed by endostatin and the ectodomain of the αvβ3 integrin that are consistent with our experimental data and with data from the literature. The headpiece of this integrin, which contains the ligand-binding site, consists of a seven-bladed β-propeller domain of the αv subunit and a βA domain looping out from a unique Ig-like “hybrid” domain in β3. SPR inhibition data and molecular modeling of endostatin-αvβ3 integrin complex suggest that although it lacks a RGD sequence, endostatin binds to the extracellular domain of integrin on the same site as the RGD peptide. Like αvβ3 integrin, α5β1 and αvβ5 integrins are RGD-recognizing integrins, and it is likely that endostatin binds to these integrins at the RGD-binding site. This is supported by the fact that recombinant endostatin binds to α5β1 integrin in a RGD motif-dependent manner and competes for the RGD-binding sites within fibronectin (36). Because RGD peptides failed to block the endostatin-α5β1 interaction in a solid phase binding assay, it is possible that endostatin, in addition to binding to the classical RGD-binding site in an integrin, also interacts with another, yet-to-be-determined site (12).

In both models five (Asp179, Thr182, Arg214, Glu220, Asp251) of the nine residues of the β3 subunit predicted to contact endostatin are identical. Two of these residues, Asp179 and Thr182, are within the β3 sequence Asp179–Thr183 reported to bind the RGD peptide in the extracellular domain of αvβ3 (29). Endostatin is also in contact with Glu220, which belongs to the metal ion-dependent adhesion site (28). In the αv subunit, four residues of the four predicted to contact endostatin in the first model, and four of six predicted to contact endostatin in the second model were identical (Ile147, Asp148, Asp150, and Gln214). This is in agreement with the predicted ligand-binding residues on the αv subunit (Arg143, Phe154) (29).

Six arginine residues were predicted to be in contact with the αvβ3 integrin in the first model, and five were predicted in the second model. In the first predicted model, two arginine residues Arg63 and Arg66 belonging to the ES-2 peptide (an arginine-rich peptide encompassing residues 60–70 of endostatin (15) are in contact with the αv subunit. In the second model, the ES-2 peptide is in contact neither with the αv subunit, nor with the β3 subunit. When immobilized, this peptide mediates endothelial cell adhesion (16). Because the adhesion was inhibited by heparin and by antibodies against β1 integrin, it has been reported that this peptide binds to integrins (16). However, the binding of the ES-2 peptide to β1 integrins could occur at a site distinct from the RGD ligand-binding pocket (16), and it is difficult to discriminate between the two models without further data. Several arginine residues of endostatin (Arg24, Arg3, and Arg139) participate in the binding to the αvβ3 integrin in both models and to heparan sulfate (9, 28). These results suggest that the αvβ3 integrin and heparan sulfate bind to the same site, or to substantially overlapped sites, on endothelial and that endostatin is not able to bind simultaneously to the αvβ3 integrin and heparan sulfate. The double R27A/R139A mutant has lost its ability to bind integrins. Arg27 is involved in hydrogen bonding in the first model, whereas both Arg27 and Arg139 residues participate in hydrogen bonding in the second model. Furthermore, computational alanine scanning predicts that mutation of Arg27 and Arg139 into alanine would destabilize the endostatin-integrin complex in the second and first model, respectively, with Arg27 being the fourth hottest spot and Arg139 the third hottest spot. These results support the major role played by these two arginine residues in the binding of endostatin to αvβ3 integrin.

In the two models of the endostatin-integrin complex, the amino acid residues predicted to destabilize the complex are identical in the αv subunit, whereas there is only a single amino acid (Tyr132) of the β3 subunit predicted to destabilize the complex in the two models. The two models share several energetically important amino acid residues on endostatin (Arg24, Phe34, Arg7, and Arg139). The highest predicted change in binding free energy upon alanine mutation is 3.05 in model 1 and above 10 (Glu220 in the β3 subunit and Arg24 on endostatin) in model 2. Computational alanine scanning predicts that besides arginine residues, Phe34 and Phe32 would destabilize the
endostatin-integrin complex in model 2, whereas only Phe\textsuperscript{34} is predicted to be energetically important in model 1. The two phenylalanine residues are exposed at the endostatin surface and have been reported to be essential for \textit{in situ} binding of endostatin to murine tissues, human endothelial cells, and immobilized laminin (37).

The D104N mutation, whether in monomeric endostatin or within the NC1 domain, did not significantly alter their ability to interact with their identified partners. This suggests that this sequence change may not be responsible for the reported high risk of occurrence of sporadic breast cancer (25) and for the influence on the age of onset of acute myeloid leukemia (26). We report in this study that two molecules present at the cell surface and able to bind to endostatin, namely integrins and heparan sulfate chains, are also able to bind to each other. Integrin-heparan sulfate interactions may play a role in endostatin signaling by contributing to its localization to lipid rafts at the surface of endothelial cells. Indeed, it has been shown that endostatin localized to lipid rafts through heparan sulfate proteoglycan and integrin-mediated interactions (15). The direct interaction between integrins and heparan sulfate at the cell surface might thus promote endostatin localization to lipid rafts. Integrin-heparan sulfate interactions are also involved in cell adhesion and seem to play a role in the spreading of CHO cells plated on the α5β1 integrin. Another integrin, the integrin αXβ2, has been reported to bind to heparin (41). The binding of α5β1 to heparin is cation-dependent, calcium being the most important, whereas the binding of the αX1 domain to heparin requires Mg\textsuperscript{2+} (41). It should be noted that the glycosaminoglycan moiety of decorin (dermatan or chondroitin sulfate) plays a key role in the interaction of α2β1 integrin with decorin (42). All of these data will contribute to a better understanding of the mechanism of endostatin binding to integrins and heparan sulfate at the surface of endothelial cells.

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