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Fibrinogen Binds to Integrin $\alpha_5\beta_1$ *via* the Carboxyl-Terminal RGD Site of the Aα-Chain[1]

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Fibrinogen interactions with vascular endothelial cells are implicated in various physiological and pathophysiological events, including angiogenesis and wound healing. We have shown previously that integrin $\alpha_5\beta_1$ is a fibrinogen receptor on endothelial cells [Suehiro, K., Gailit, J., and Plow, E.F. (1997) *J. Biol. Chem.* 272, 5360–5366]. In the present study, we have characterized fibrinogen interactions with purified $\alpha_5\beta_1$ and have identified the recognition sequence in fibrinogen for $\alpha_5\beta_1$. The binding of fibrinogen to immobilized $\alpha_5\beta_1$ was selectively supported by Mn^{2+}. Fibrinogen bound to purified $\alpha_5\beta_1$ in a time-dependent, specific, and saturable manner in the presence of Mn^{2+}, and the binding was blocked completely by Arg-Gly-Asp (RGD)–containing peptides and by anti-α_5 and anti-$\alpha_5\beta_1$ monoclonal antibodies. A monoclonal antibody directed to the C-terminal RGD sequence at Aα572–574 significantly inhibited the binding of fibrinogen to $\alpha_5\beta_1$, whereas monoclonal antibodies directed to either the N-terminal RGD sequence at Aα95–97 or the C-terminus of the γ-chain did not. Furthermore, substituting RGE for RGD at position Aα95–97 in recombinant fibrinogen had a minimal effect on binding, whereas substituting RGE for RGD at position Aα572–574 decreased binding by 90%. These results demonstrate that the C-terminal RGD sequence at Aα572–574 is required for the interaction of fibrinogen with $\alpha_5\beta_1$.

Key words: divalent cations, fibrinogen, fibronectin receptor, integrin, RGD.

Endothelial cells possess many anti-coagulant and anti-thrombotic functions on their luminal surface. When endothelial cells are disrupted and subendothelium is exposed, it results in local thrombus formation, and accumulation of fibrinogen or fibrin is observed at such sites of vascular injury. It has been demonstrated that fibrinogen can induce attachment, spreading and migration of endothelial cells (*1*, *2*), and the interactions of fibrinogen/fibrin with endothelial cells have been implicated in a wide variety of physiological and pathophysiological circumstances such as angiogenesis and wound healing (*3–6*).

Several distinct receptors have been found to mediate fibrinogen binding to endothelial cells. These include $\alpha_v\beta_3$ (*7*, *8*), the vitronectin receptor, which is a member of the integrin family of cell adhesion receptors, as well as several non-integrin binding sites such as intercellular adhesion molecule–1 (*9*).

Fibrinogen interacts with $\alpha_v\beta_3$ *via* an Arg-Gly-Asp (RGD) recognition specificity (*10*). This tripeptide sequence is recognized not only by $\alpha_v\beta_3$ but also by many other integrins (*11–13*), including $\alpha_5\beta_1$, which serves as a fibronectin receptor on endothelial cells and many other cell types (*14–17*). Fibrinogen contains two RGD sequences within its constituent Aα-chain: RGDF at Aα95–98 and RGDS at Aα572–575. Previous studies employing recombinant fibrinogens with mutations at these RGD sequences have shown that endothelial cells bind to immobilized fibrinogen *via* the RGD sequence at Aα572–574 (*18*).

The recognition specificity of integrins is regulated by divalent cations. For example, Mn^{2+} supports fibrinogen binding to $\alpha_v\beta_3$, whereas Ca^{2+} antagonizes this binding (*19*, *20*). This pattern of cation regulation is not unique to β_3 integrins; ligand binding to β_1 and β_2 integrins is also regulated by divalent cations (*21–26*). Recently, we identified $\alpha_5\beta_1$ as a novel receptor which mediated fibrinogen binding to human umbilical vein endothelial cells. This $\alpha_5\beta_1$-mediated interaction of fibrinogen with endothelial cells was not supported by Ca^{2+} or Mg^{2+}, but was supported specifically by Mn^{2+} (*27*). In the present study, we have characterized the direct interaction of fibrinogen with $\alpha_5\beta_1$ specifically supported by Mn^{2+} by using purified $\alpha_5\beta_1$ and have identified the recognition sequence in fibrinogen that participates in its interaction with purified $\alpha_5\beta_1$. By employing anti-peptide monoclonal antibodies (mAbs) specific for each of the RGD sequences in the fibrinogen Aα-chain and recombinant fibrinogens with mutations at these RGD sequences,

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Abbreviations: BSA, bovine serum albumin; mAb, monoclonal antibody; rFg, recombinant fibrinogen; RGD, Arg-Gly-Asp; TBS, Tris-buffered saline.

we showed that the C-terminal RGD sequence at Aα572–574 is involved in the interaction of fibrinogen with $\alpha_5\beta_1$.

MATERIALS AND METHODS

Purification of Proteins—Plasma-derived fibrinogen was purified by affinity chromatography using an anti-fibrinogen mAb column. Ca^{2+}-dependent anti-fibrinogen mAb IF-1 (*28*) was purchased from Iatron Laboratories (Tokyo), and was conjugated to CNBr-activated Sepharose 4B. Human fibrinogen obtained by a glycine precipitation method (The Chemo-Sero-Therapeutic Research Institute, Kumamoto) (*29*) was dissolved in Tris-buffered saline (TBS) to 2 mg/ml, supplemented with 2 mM $CaCl_2$, and was applied to the IF-1–conjugated Sepharose 4B column. After washing, fibrinogen was eluted with TBS containing 5 mM EDTA and dialyzed against TBS. The contamination of fibronectin in this fibrinogen preparation was 0.06% (w/w) by ELISA assay. When the specific binding of a biotin-labeled 120-kDa chymotryptic fibronectin fragment to purified $\alpha_5\beta_1$ was measured in the presence of 1 mM Mn^{2+}, the apparent dissociation constant (K_d) was 10–20 nM, similar to that of fibrinogen to $\alpha_5\beta_1$. Therefore, this minor contamination of fibronectin has a negligible effect on the binding of fibrinogen to $\alpha_5\beta_1$. Fibronectin was isolated from human fresh-frozen plasma by gelatin-Sepharose chromatography (*30*). The 120-kDa chymotryptic fibronectin fragment containing the central cell-binding domain was prepared by digestion with α-chymotrypsin followed by gel filtration on Sephacryl S200 (*31*). $\alpha_5\beta_1$ was purified from human placenta as described (*21*) with some modifications. Briefly, homogenized placental tissue was washed and extracted with TBS containing 100 mM octyl-β-D-glucopyranoside (Sigma), applied to 120-kDa fibronectin fragment column in the presence of 1 mM Mn^{2+}, and the bound receptor was eluted with the buffer containing 20 mM EDTA. The eluted fractions were analyzed by SDS–polyacrylamide gel electrophoresis followed by Coomassie Blue staining and found to contain $\alpha_5\beta_1$ of more than 90% purity.

Production of Recombinant Fibrinogens—Recombinant fibrinogen molecules were designed with mutations in the putative endothelial cell-binding domains. The RGD sequences at positions Aα95–97 and Aα572–574 were mutated to RGE as described previously (*32*) to produce rFg-D97E and rFg-D574E, respectively. The double mutant rFg-D97E/D574E was made by mutating both RGD sequences at positions Aα95–97 and Aα572–574 to RGE. Recombinant fibrinogens were produced from the serum-free media of transfected baby hamster kidney cells as described in detail previously (*33*, *34*) with some modifications. Transfected baby hamster kidney cells expressing recombinant fibrinogen were cultured in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal calf serum containing antibiotics (penicillin, 50 μg/ml; streptomycin, 50 μg/ml). Cells were cultured to 80% confluence in 10-cm culture dishes, and cells from 10 dishes were collected with 0.25% trypsin/EDTA. Isolated cells were suspended in 2 liters of culture media and plated onto a Nunc Cell Factory 10 (culture area 6,320 cm^2). When the cells reached 90% confluence, the medium was replaced with serum-free Dulbecco's modified Eagle's medium (high glucose). After the cells were incubated overnight, these supernatants were harvested twice from the Cell Factory 10, and then inhibitors (5 mM ε-aminocaproic acid, 4 μM leupeptin, 0.2 mM PMSF, 1 μM pepstatin A, 50 μg/ml soybean trypsin inhibitor, 5 mM benzamidine-HCl) were added. After harvest, each conditioned medium was centrifuged at 5,000 rpm for 20 min at 4°C. The supernatants were mixed with 2 mM $CaCl_2$, and loaded for Ca^{2+}-dependent anti-fibrinogen mAb IF-1–conjugated column chromatography (*28*). The column had been equilibrated in 50 mM Tris-HCl, pH 7.2, containing 0.1 M NaCl, 5 mM ε-aminocaproic acid, 4 μM leupeptin, 0.2 mM PMSF, 1 μM pepstatin A, 50 μg/ml soybean trypsin inhibitor, 5 mM benzamidine-HCl, and 2 mM $CaCl_2$. After the harvest medium has been loaded, the column was washed with the equilibration buffer. Recombinant fibrinogens were eluted with 50 mM Tris-HCl, pH 7.2, containing 0.1 M NaCl and 10 mM EDTA. The pooled fractions were analyzed by SDS–polyacrylamide gel electrophoresis. The protein concentrations were determined by the ELISA and/or absorbance at 280 nm (A_{280} = 16.0 for 1% fibrinogen) after dialysis to remove EDTA.

Peptides and Antibodies—The synthetic peptides GRGDSP and GRGESP were purchased from Life Technologies (Rockville, MD) and the peptide GQQHHLGGAKQAGDV from the extreme C-terminus of fibrinogen γ-chain was from Bachem AG (Bubendorf, Switzerland). MAb 7E3 (*35*), which recognizes β_3 integrins, was provided by Dr. Barry S. Coller, Mount Sinai School of Medicine, New York, NY. MAbs LM609 (*8*) directed to $\alpha_v\beta_3$, JBS5 (*36*) directed to $\alpha_5\beta_1$, MAB 1980 (*37*) directed to α_v, and JB1a, originally designated J10 (*38*), directed to β_1 were purchased from Chemicon International (Temecula, CA). MAb P1D6 (*39*) against α_5 was obtained from Life Technologies. Two mAbs raised against RGD-containing synthetic peptides from the fibrinogen Aα-chain, anti-N directed to N-terminal RGD sequence Aα87–100 (TTNIMEILRGDFSS) and anti-C directed to C-terminal RGD sequence Aα566–580 (SSTAYNRGDSTFESK) (*40*), were provided by Dr. Zaverio M. Ruggeri, The Scripps Research Institute, La Jolla, CA. MAb 4A5 (*41*), which recognizes the C-terminus of the γ-chain of fibrinogen, was purchased from Boston Research Services (Winchester, MA).

Solid-Phase Ligand Binding Assay—The binding of fibrinogen or the 120-kDa chymotryptic fibronectin fragment to purified and immobilized $\alpha_5\beta_1$ was performed as described (*26*) with some modifications. Plasma-derived fibrinogen, recombinant fibrinogens and the 120-kDa chymotryptic fibronectin fragment were labeled with sulfo-NHS biotin (Pierce, Rockford, IL). $\alpha_5\beta_1$ (1.0 mg/ml) was diluted 1:500 with 150 mM NaCl, 20 mM Tris, pH 7.4 (Buffer A), immobilized in 96-well Immulon-3 microtiter plates (Dynatech Laboratories, Chantilly, VA) at 200 ng per well, and incubated overnight at 4°C. After the plates were blocked with 1% (w/v) BSA in Buffer A, biotin-labeled ligands were added (20 nM) in Buffer A containing 1 mg/ml BSA and the selected divalent ions at 1 mM concentrations, and incubated for 180 min at 37°C. All buffers were pretreated with Chelex 100 (Bio-Rad Laboratories, Hercules, CA) to remove undesired cations. Bound ligand was quantified by the addition of a 1:200 dilution of ExtrAvidin-peroxidase conjugate (Sigma) in Buffer A containing 1 mg/ml BSA for 10 min at room temperature, the color was developed using ABTS substrate, and the absorbance at 405 nm was measured. Non-specific binding was measured by determining the ligand binding to wells coated with BSA

alone at each cation condition, and these values were subtracted from the corresponding values for receptor-coated wells. Effects of synthetic peptides or mAbs were tested by simultaneously adding these inhibitors to microtiter wells in the presence of 1 mM Mn^{2+}. An apparent dissociation constant (K_d) was calculated using the equation $A = A_{max} \times \{[L] / (K_d + [L])\}$, where A is the absorbance at 405 nm, A_{max} is the maximum absorbance at saturation, and [L] is the molar concentration of free ligand. K_d values were derived by non-linear regression analyses of specific binding isotherms using the program DeltaGraph Version 4.5 for Macintosh (DeltaPoint). Data were determined as the mean of triplicate measurements at each experimental point.

RESULTS

Manganese Supports Fibrinogen Binding to Purified $\alpha_5\beta_1$—To demonstrate the direct binding of fibrinogen to $\alpha_5\beta_1$, fibrinogen was labeled with biotin, and its interaction with purified and immobilized $\alpha_5\beta_1$ was measured. Using the biotin-labeled 120-kDa chymotryptic fibronectin fragment as a test ligand, substantial binding of this ligand to immobilized $\alpha_5\beta_1$ was observed in the presence of Mn^{2+} but not in the presence of Ca^{2+}. This interaction exhibited an appropriate specificity profile as it was more than 95% inhibited by an RGD-containing peptide (GRGDSP) and by anti-$\alpha_5\beta_1$ mAb (data not shown). Next, biotin-labeled fibrinogen binding to $\alpha_5\beta_1$ was performed under the same conditions. This binding was also divalent cation–dependent and was inhibited by EDTA. To assess how divalent cations affect the interaction, biotin-labeled fibrinogen was incubated with immobilized $\alpha_5\beta_1$ in the presence of various concentrations of different cations for 3 h at 37°C. As shown in Fig. 1, this binding was selectively supported by Mn^{2+}, whereas Mg^{2+} and Ca^{2+} failed to enhance the binding in the range of 10 μM to 1 mM. In subsequent experiments, 1 mM Mn^{2+} was chosen as a concentration which supported extensive fibrinogen binding.

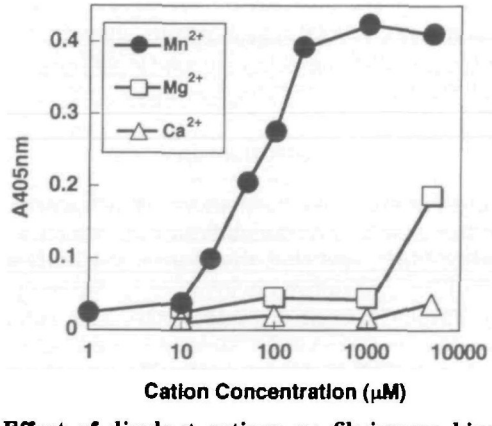

Fig. 1. **Effect of divalent cations on fibrinogen binding to $\alpha_5\beta_1$.** Biotinylated-fibrinogen (20 nM) was added to microtiter wells coated with purified $\alpha_5\beta_1$ and incubated for 3 h at 37°C in the presence of various concentrations of divalent cations. Bound fibrinogen was quantified by the addition of ExtrAvidin-peroxidase conjugate, the color was developed using ABTS substrate, and the absorbance at 405 nm was measured. The results shown are the average of triplicate measurements performed at each experimental data point and are representative of three separate experiments.

To establish the specificity of fibrinogen binding to $\alpha_5\beta_1$ in the presence of Mn^{2+}, several synthetic peptides were tested (Fig. 2A). An RGD-containing peptide, GRGDSP, inhibited binding by 95%, while the control peptide, GRGESP, had no effect on the interaction. The peptide from the C-terminus of the fibrinogen γ-chain (residues 397–411), which effectively blocked fibrinogen binding to $\alpha_{IIb}\beta_3$, had no effect. The effects of function-blocking anti-integrin mAbs were also tested (Fig. 2B). The binding was significantly inhibited by anti-β_1 mAb (54%) and was completely blocked by anti-α_5 and anti-$\alpha_5\beta_1$ mAbs (95 and 96%, respectively).

Characterization of Fibrinogen Binding to $\alpha_5\beta_1$—The time course of the binding of fibrinogen to $\alpha_5\beta_1$ in the presence of Mn^{2+} was examined over a 6-h time course with the fibrinogen concentration at 20 nM. Non-specific binding was determined as the binding to wells coated with BSA. Specific binding reached a constant level at 3 h (data not shown). In subsequent experiments, 3 h was selected as the incubation time. The specific binding of fibrinogen to purified $\alpha_5\beta_1$ in the presence of 1 mM Mn^{2+} was saturable (Fig. 3). Using non-linear regression analysis, changes in fibrinogen binding fit well to occupancy of a single class of high affinity binding sites, and an apparent dissociation constant (K_d) was calculated to be 12.7 ± 1.9 nM (n = 4).

Localization of the $\alpha_5\beta_1$ Recognition Sequence in Fibrinogen—The RGD sensitivity of fibrinogen binding to $\alpha_5\beta_1$ suggests that one of the two RGD sequences in the Aα-chain is involved in receptor recognition. To identify which RGD

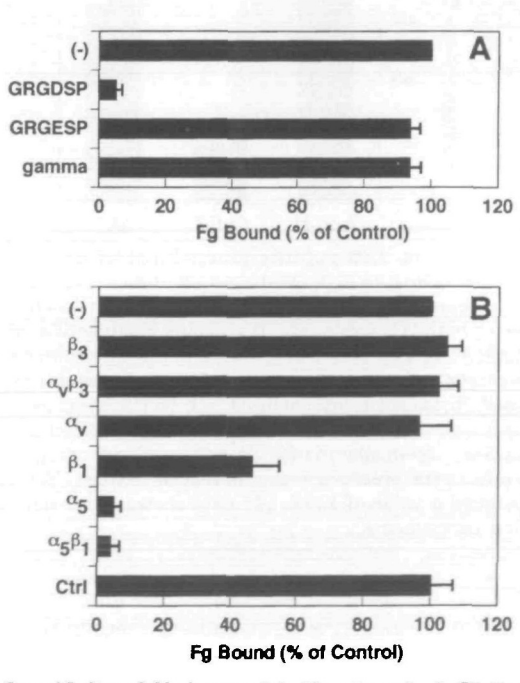

Fig. 2. **Specificity of fibrinogen binding to $\alpha_5\beta_1$.** A: Biotinylated-fibrinogen (20 nM) was incubated with immobilized $\alpha_5\beta_1$ in the presence of GRGDSP (100 μM), GRGESP (100 μM), and γ-chain peptide GQQHHLGGAKQAGDV (100 μM). B: Fibrinogen binding to $\alpha_5\beta_1$ was also tested in the presence of anti-β_3 mAb 7E3 (20 μg/ml), anti-$\alpha_v\beta_3$ mAb LM609 (20 μg/ml), anti-α_v mAb MAB1980 (20 μg/ml), anti-β_1 mAb JB1a (1/200 dilution), anti-α_5 mAb P1D6 (1/200 dilution), anti-$\alpha_5\beta_1$ mAb JBS5 (1/200 dilution), or control mouse ascites (Ctrl.) (1/200 dilution). In both panels, incubation was done for 3 h at 37°C in the presence of 1 mM Mn^{2+}.

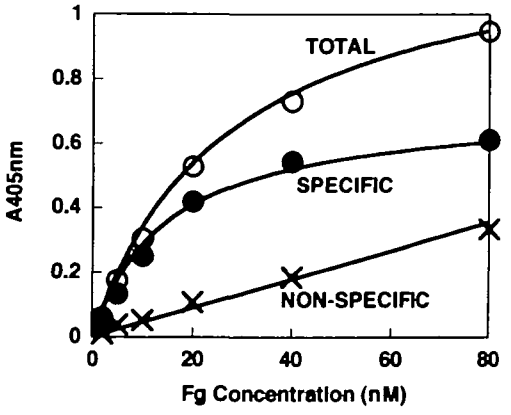

Fig. 3. **Saturation isotherm of fibrinogen binding to $\alpha_5\beta_1$.** Binding isotherms of fibrinogen to $\alpha_5\beta_1$ were constructed by incubating increasing concentrations of biotinylated-fibrinogen with immobilized $\alpha_5\beta_1$ in the presence of 1 mM Mn^{2+}. Specific binding was calculated by subtracting non-specific binding, measured as the binding to wells coated with BSA, from the total binding. The data shown are the average of triplicate measurements at each experimental data point and are representative of four separate experiments. The apparent dissociation constant (K_d) was 12.7 ± 1.9 nM (n = 4).

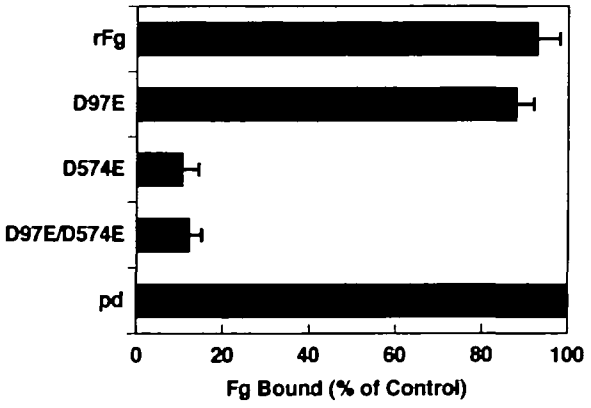

Fig. 5. **Recombinant fibrinogen binding to $\alpha_5\beta_1$.** Biotinylated recombinant fibrinogens rFg (recombinant fibrinogen wild type), D97E (recombinant fibrinogen with a mutation of RGE for RGD at Aα95–97), D574E (recombinant fibrinogen with a mutation of RGE for RGD at Aα572–574), and D97E/D574E (recombinant fibrinogen with mutations of RGE for RGD at both Aα95–97 and Aα572–574) at 20 nM were incubated for 3 h at 37°C with immobilized $\alpha_5\beta_1$ in the presence of 1 mM Mn^{2+}. The binding of plasma-derived fibrinogen (pd) to $\alpha_5\beta_1$ was assigned a value of 100%.

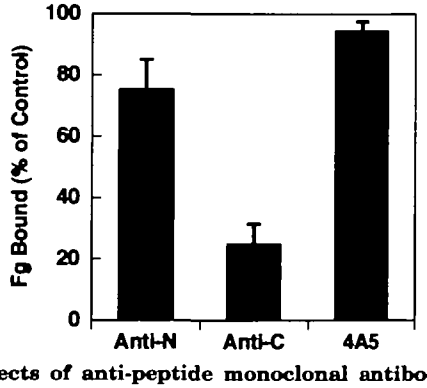

Fig. 4. **Effects of anti-peptide monoclonal antibodies on fibrinogen binding to $\alpha_5\beta_1$.** MAbs anti-N (directed to the N-terminal RGD sequence residues 87–100 of the fibrinogen Aα-chain), anti-C (directed to the C-terminal RGD sequence residues 566–580 of the fibrinogen Aα-chain), and 4A5 (directed to the C-terminal region of the fibrinogen γ-chain residues 395–411) were tested. Binding of biotinylated-fibrinogen to immobilized $\alpha_5\beta_1$ in the presence of 1 mM Mn^{2+} was measured by simultaneously adding the selected mAbs (1/200 dilution) and incubating for 3 h at 37°C. The binding of fibrinogen to $\alpha_5\beta_1$ in the presence of control mouse ascites (1/200 dilution) was assigned a value of 100%. The data shown are means and SD from four experiments.

sequence in fibrinogen is involved in the recognition of $\alpha_5\beta_1$, we tested two mAbs which specifically recognize one of the two RGD sequences in the Aα-chain. As shown in Fig. 4, mAb anti-C, directed to the C-terminal RGD sequence of the Aα-chain of fibrinogen, significantly inhibited (75%) the binding of fibrinogen to $\alpha_5\beta_1$, as compared to mAb anti-N (25%), directed to the N-terminal RGD sequence. MAb 4A5 (reactive to the C-terminus of the γ-chain of fibrinogen), which effectively blocked fibrinogen binding to $\alpha_{IIb}\beta_3$, had minimal effect on this interaction (Fig. 4). This result suggests that $\alpha_5\beta_1$ interacts with the C-terminal RGD sequence in its recognition of fibrinogen.

Recombinant Fibrinogen Binding to $\alpha_5\beta_1$—We tested recombinant fibrinogens (rFgs) with a mutation at one or both of the two RGD sequences in the Aα-chain to further clarify which RGD sequence in fibrinogen is involved in the recognition of $\alpha_5\beta_1$. The purified recombinant fibrinogens showed the proper complement of Aα-, Bβ-, and γ-chains on reduced SDS–polyacrylamide gel electrophoresis as reported (*18*, *32*). rFg-D97E, with a substitution at the N-terminal RGD sequence, and rFg-D574E, with a substitution of RGE for RGD at the C-terminal RGD sequence, were tested. As shown in Fig. 5, rFg-D574E showed only minimal binding (10%) to $\alpha_5\beta_1$ compared to rFg-D97E, which showed similar binding (88%) to rFg wild type (93%) or plasma-derived fibrinogen. rFg-D97E/D574E with mutations at both RGD sequences showed only minimal binding (12%), as did rFg-D574E. These results show that the functional region in fibrinogen that interacts with $\alpha_5\beta_1$ is the C-terminal RGD sequence of the Aα-chain.

DISCUSSION

In the present study, we have shown that fibrinogen binds directly to purified and immobilized $\alpha_5\beta_1$ selectively in the presence of Mn^{2+}, and that the C-terminal RGD sequence at Aα572–574 of fibrinogen is involved in the recognition of $\alpha_5\beta_1$ by employing anti-peptide mAbs and recombinant fibrinogens.

The ligand-binding function of $\alpha_5\beta_1$ was dramatically altered by divalent cations: fibrinogen binding to purified $\alpha_5\beta_1$ was supported by Mn^{2+} but not by Ca^{2+}or Mg^{2+}. It is generally accepted that divalent cation occupancy and ligand binding in the integrin are intimately linked, and both integrin α and β subunits have been implicated in divalent cation as well as ligand binding (*42*, *43*). This cation regulation of ligand binding may be explained by the hypothesis that conformational change in the integrin induced by Mn^{2+} may be more favorable for ligand binding

(*25*, *44*). A study using function-perturbing mAbs to $\alpha_5\beta_1$ (*45*), which revealed that each divalent cation has distinct effects on the conformational change in the integrin that results in exposure of the ligand-binding sites, may support this hypothesis.

We employed recombinant fibrinogens for the determination of the recognition sequence in fibrinogen to interact with $\alpha_5\beta_1$. Recombinant fibrinogens have been used to identify the binding sites in fibrinogen that interact with several integrins. In fibrinogen interaction with $\alpha_{IIb}\beta_3$ on platelets, three potential binding sites had been proposed: two on the RGD sequences in the Aα-chain and one on the C-terminus of the γ-chain. Although studies using fibrinogen fragments, synthetic peptides and mAbs were inconclusive regarding the importance of these three sites (*40*, *46*, *47*), recombinant fibrinogens with mutations at one of these sites have clearly identified that the C-terminus of the γ-chain is critical. Recombinant fibrinogens have also been useful for the determination of the binding site in fibrinogen that interacts with $\alpha_v\beta_3$ on vascular endothelial cells (*18*).

The recognition of fibrinogen by $\alpha_5\beta_1$ demonstrated in this study may be relevant in a wide variety of physiological and pathophysiological conditions. Wound healing at sites of vascular injury may be an illustrative example. As fibrinogen or fibrin is present at sites of vascular injury, direct contact of $\alpha_5\beta_1$-bearing endothelial cells with fibrinogen may facilitate re-establishment of a non-thrombogenic surface on the injured vessel wall. As recent reports showed that retraction of fibrin clots by smooth muscle cells was mediated by $\alpha_5\beta_1$ (*48*, *49*), the interaction of fibrinogen/fibrin with $\alpha_5\beta_1$-bearing smooth muscle cells in the injured vessel wall may be critical to vascular narrowing of the atherosclerotic vessels. Therefore, fibrinogen interactions with $\alpha_5\beta_1$ may have a significant role in many conditions such as wound healing and atherosclerosis.

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