A Unique Recognition Site Mediates the Interaction of Fibrinogen with the Leukocyte Integrin Mac-1 (CD11b/CD18)*

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Mac-1 (CD11b/CD18), a leukocyte-restricted integrin receptor, mediates neutrophil-monocyte adhesion to vascular endothelium and phagocytosis of complement-opsonized particles. Recent studies have shown that Mac-1 also functions as a receptor for fibrinogen in a reaction linked to fibrin deposition on the monocyte surface. In this study, we have used extended proteolytic digestion of fibrinogen to identify the region of this molecule that interacts with Mac-1. We found that an $M_r$ ~30,000 plasmic fragment D of fibrinogen (D30) produced dose-dependent inhibition (IC50 = 1.6 $\mu$M) of the interaction of intact 128I-fibrinogen with stimulated neutrophils and monocytes. D30 bound saturably to these cells with specific association of 136,200 $\pm$ 15,000 molecules/cell in a reaction inhibited by OKM1 and M1/70, monoclonal antibodies specific for the $\alpha$ subunit of Mac-1. Direct microsequence analysis and an epitope-mapped monoclonal antibody showed that D30 lacks the COOH-terminal dodecapeptide of the $\gamma$ chain as well as the Arg-Gly-Asp sequences in the $\alpha$ chain. We conclude that fibrinogen interacts with the leukocyte integrin Mac-1 through a novel recognition site that is not shared with other known integrins that function as fibrinogen receptors.

The deposition of fibrinogen on the leukocyte surface is a hallmark of a variety of inflammatory responses (1). Fibrinogen association with monocytes or neutrophils has in fact been recognized as a causal component of delayed type hypersensitivity (2, 3), incompatible transplant rejection (4), and in the physiopathology of vascular obstruction and athereogenesis (5-8).

In more recent studies, the interaction of fibrinogen with leukocytes has been postulated as an example of a dynamic cell adhesion reaction, involving a specific and coordinated receptor-mediated recognition (9-12). In this context, broad mechanisms of cell adhesion involving both cell-cell communication as well as interactions with extracellular matrix proteins have been shown to be regulated by specialized membrane receptors expressed on virtually all cells (13). These highly homologous molecules that have been termed integrins (14) are heterodimeric transmembrane glycoproteins composed of noncovalently associated $\alpha/\beta$ subunits (14) and, in many cases, are functionally characterized by their versatile ability to recognize multiple ligands (15) through the Arg-Gly-Asp (RGD) sequence (16, 17).

Integrin receptors on platelets ($\alpha_{IIb}/\beta_{3}$) or endothelial cells ($\alpha_{IIb}/\beta_{3}$) mediate the interaction with fibrinogen through the RGD sequence (15, 16-18), and receptor occupancy contributes to a complex hemostatic response involving platelet aggregation (19), adhesion (20), and integrity of the vessel wall (21). Also the integrin Mac-1 (CD11b/CD18) (14), expressed on monocytes and neutrophils (22), binds fibrinogen (23-26) in a reaction functionally linked to inflammation and local deposition of fibrin (23-26).

In this study we sought to determine the molecular requirements supporting the interaction of fibrinogen with the leukocyte integrin Mac-1 (14, 22-26). We have found that Mac-1 binds fibrinogen through a novel interacting region in the ligand. This recognition site is unique as compared with fibrinogen binding to other integrins and is contained within an $M_r$ ~30,000 plasmic fragment D that lacks the RGD sequences in the $\alpha$ chain as well as the COOH-terminal dodecapeptide of the $\gamma$ chain.

MATERIALS AND METHODS

Cells and Cell Culture—Polymorphonuclear leukocytes (PMN)* were prepared by dextran sedimentation from acid citrate/dextran anticoagulated blood and preparatively depleted of platelets by centrifugation at 800 $\times$ g for 15 min and of mononuclear cells by differential centrifugation over Ficoll-Hypaque (Sigma). Cells were resuspended at 1.5 $\times$ 10$^7$ cells/ml in RPMI 1640 medium (Invitrogen Scientific) supplemented with 20 mM Hepes (Calbiochem), kept in ice, and used within 2 h of isolation. The monocyte cell line THP-1 (American Type Culture Collection) was maintained in continuous culture in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (Gemini Bioproducts), 20 mM Hepes, 2 mM l-glutamine (Irvin), and 10 mM 2-mercaptoethanol (Eastman Kodak). Cells were harvested, washed twice in ice-cold phosphate-buffered saline supplemented with 5 mM EDTA, and resuspended in serum-free RPMI 1640 at 1.5 $\times$ 10$^7$/ml.

Protein Characterization and Labeling—The synthetic peptides Arg-Gly-Asp-Ser (RGDS) and the dodecapeptide H12 (which duplicates the COOH terminus of the fibrinogen $\gamma$ chain) were synthesized and characterized as described (27, 28). The experimental procedures for the purification and characterization of plasma fibrinogen have been described elsewhere (29). Proteolytic digestion of fibrinogen was carried out according to published methods (29, 30). Briefly, 50 mg of fibrinogen in 0.01 M Tris-HCl, 0.14 M NaCl, pH 7.4 (TBS), were incubated with streptokinase-activated plasminogen (18 $\mu$g/ml) in the presence or in the absence of 2 mM urea for various time intervals at 37°C. Each reaction was terminated by the addition of 50,000 units/ml Trasylol (Sigma), followed by extensive dialysis, and separation of the fragments by chromatography on Sephadex G-100.
(Pharmacia LKB Biotechnology Inc.). Fibrinogen fragment D (M, \(\sim 80,000\)) was proteolytically digested with plasmin with or without 2 M urea for 24 h at 37 °C, extensively dialyzed against TBS, and the resulting species isolated to >90% homogeneity by HPLC using a Mono-Q column (Pharmacia) equilibrated in 0.01 M NaPO₄, pH 7.0, with elution in 0.01 M NaPO₄, 1 M NaCl, pH 7.0. This 24-h plasmic digest of fragment D migrated with an apparent Mₚ of 30,000 on 15% SDS-polyacrylamide gels under nonreducing conditions (Dₚₜ) and was comprised of a tightly spaced doublet of Mₚ = 13,000 and 11,000 and a lower molecular weight constituent upon reduction. In other experiments, 90-well flat bottomed ELISA plates (Costar) were separately coated with increasing concentrations (0.1–10 μg/ml) of fibrinogen or fibrinogen fragments X, D:E, and D for 16 h at 4 °C. After washing to remove further saturation of nonspecific binding sites with phosphate-buffered saline containing 0.05% Tween, 1% bovine serum albumin, and 0.02% Na₂PO₄, pH 7.4, plates were incubated with 10 μg/ml monoclonal antibody 4A5, specific for the COOH terminus of the fibrinogen γ chain (31), for an additional 2 h at 37 °C. After washes, binding of monoclonal antibody 4A5 was revealed by the addition of a 1:250 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG, followed by 1 mg/ml p-nitrophenyl phosphate (disodium) in NaHCO₃ buffer, pH 9.8. Optical density was determined at 405 nm on a Vmax 96-well plate ELISA reader. Protein sequencing was carried out as described previously (32). Fibrinogen and fragment Dₚₜ were iodinated by the IODO-GEN method (33) to a specific activity of 7.03 and 4.5 X 10⁶ cpm/μg, respectively.

**Bind Reactions**—The experimental procedures for measuring the inducible binding of ¹²⁵I-fibrinogen to leukocytes have been described previously in detail (12, 23). Briefly, PMN or THP-1 cells at 1.5 X 10⁶/ml were suspended in serum-free RPMI 1640 in the presence of 2.5 mM CaCl₂ and 100 μM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK, Calbiochem). Cells were stimulated with 10 μM of the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma) immediately before the addition of increasing concentrations of the ¹²⁵I-labeled ligand (fibrinogen or Dₚₜ). The reaction was terminated after a 20-min incubation at room temperature by centrifugation of aliquots of the cell suspensions through a mixture of silicone oils at 12,000 X g for 5 min at room temperature to separate free from cell-associated radioactivity. Nonspecific binding was assessed in the presence of a 100-fold molar excess of unlabeled protein (fibrinogen or Dₚₜ) added at the start of the incubation and was subtracted from the total binding to calculate specific binding. For competition experiments, fMLP-stimulated PMN or THP-1 cells were simultaneously incubated with a saturating dose of ¹²⁵I-fibrinogen and increasing concentrations of the various competing proteins for 20 min at room temperature. In separate experiments, aliquots of PMN or THP-1 cells were preincubated with saturating concentrations of various anti Mac-1 (CD11b/CD18) monoclonal antibodies specific for either the α (OKM1, M1/70, 60.1) or β (60.3, IB4) subunit for 30 min at room temperature. Cells were then stimulated with 10 μM fMLP in the presence of 2.5 mM CaCl₂ and equilibrated with a fixed concentration of ¹²⁵I-fibrinogen (50 μg/ml) or ¹²⁵I-Dₚₜ (5 μg/ml) for an additional 20 min at room temperature. Control monoclonal antibody used for these inhibition experiments was the anti-class I MHC W6/32.

Quantitative parameters for the interaction of ¹²⁵I-Dₚₜ with stimulated PMN were estimated by the LIGAND program. Data for the inhibition of ¹²⁵I-fibrinogen binding to fMLP-stimulated PMN mediated by fragment Dₚₜ were analyzed by the Lineweaver-Burk double-reciprocal plot.

**RESULTS AND DISCUSSION**

Previous studies have shown that the leukocyte integrin Mac-1 (CD11b/CD18) (14, 22) functions as a receptor for fibrinogen on monocytes, PMN, and various transformed cell lines of myelomonocytic lineage (23–26).

In preliminary experiments, various proteolytic fragments derived from sequential plasmic digestion of fibrinogen (29, 30) were tested for their ability to inhibit the binding of intact ¹²⁵I-fibrinogen to stimulated PMN or monocyte THP-1 cells. As previously reported, THP-1 cells provide a suitable in vitro model of the monocyte phenotype with respect to surface expression of Mac-1 (CD11b/CD18) and for the inducible high affinity ligand recognition of this receptor (23). The results of these experiments are summarized in Fig. 1. Fibrinogen fragments X, D:E, and D inhibited the binding of ¹²⁵I-fibrinogen to stimulated PMN in a concentration-dependent fashion with IC₅₀ ranging between 25 and 50 μg/ml added competitor (Fig. 1). Under the same experimental conditions, 1 mM concentrations of the synthetic peptides RGDS and H12 alone or in combination did not inhibit ¹²⁵I-fibrinogen binding to PMN, in agreement with previous observations (23) (Fig. 1).

These inhibitory fibrinogen fragments were further analyzed for their reactivity with the epitope-mapped monoclonal antibody 4A5, specific for the COOH-terminal dodecapeptide of the fibrinogen γ chain (31). This region plays a crucial role in mediating the interaction of fibrinogen with the platelet integrin α₅β₃ (28, 34, 35) and was also previously implicated in Mac-1 recognition of fibrinogen (24). As shown in Fig. 2, monoclonal antibody 4A5 bound specifically to fibrinogen, fragment X, and D:E while it failed to react with the Mₚ, ~80,000 fragment D (Fig. 2B). In contrast, a rabbit polyclonal antibody directed to the region 95–264 of the γ chain (29) strongly reacted with fragment D under comparable experimental conditions (not shown), thus verifying the authenticity of this fragment. Proteolytic cleavage to delete the COOH terminus of the γ chain is a well-established structural characteristic of the plasmic-derived Mₚ, ~80,000 fragment D of fibrinogen (36–38). On the other hand, these experiments raise the possibility that fibrinogen interaction with the leukocyte integrin Mac-1 occurred through a novel recognition site, structurally distinct from the previously described regions that mediate fibrinogen binding to platelet or endothelial cell integrins (15, 18, 27, 28, 34, 35, 39, 40). To further investigate this possibility, the inhibitory Mₚ, ~80,000 fragment D was subjected to 24-h plasmic proteolysis to originate an Mₚ, ~30,000 advanced fragment D (Dₚₜ) (30) that was isolated by ion exchange chromatography. Increasing concen-

![Fig. 1. Effects of fibrinogen or various fibrinogen fragments on ¹²⁵I-fibrinogen binding to PMN.](image-url)
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concentrations of D90 also inhibited 125I-fibrinogen binding to fMLP-stimulated THP-1 cells in a dose-dependent manner (IC50 = 50 μg/ml). Analysis of the binding data by the Line-weaver-Burk double-reciprocal plot indicated that D90 behaves as a competitive inhibitor of 125I-fibrinogen binding to PMN (y = 1.98 x 10^6 fibrinogen without competitor; y' = 2.57 x 10^6 fibrinogen in the presence of D90).

The molecular prerequisite for the inhibition of Mac-1-fibrinogen interaction mediated by D90 was further established in direct 125I-labeled binding experiments. As shown in Fig. 4, suspensions of PMN bound 125I-D90 in a specific and saturable reaction, competitively inhibited by a molar excess of unlabeled fibrinogen or D90 and approaching steady state at 15–20 μg/ml added protein, with association of ~136,200 ± 15,000 molecules of D90/cell (Fig. 4). Binding of 125I-D90 occurred with quantitatively identical characteristics to THP-1 cells (not shown), and as previously described for the binding of the intact fibrinogen molecule (12, 23), it required cell stimulation (10 μM fMLP) and divalent cations (2.5 mM CaCl2).

Finally, the 125I-labeled cell-bound material showed a molecular size and structural organization indistinguishable from the D90 fragment when analyzed by SDS-gel electrophoresis and autoradiography under reducing or nonreducing conditions (Fig. 4, inset).

The possible role of the leukocyte fibrinogen receptor Mac-1 (23–26) in mediating the inducible recognition of D90 was investigated further using monoclonal antibody strategy. Saturating concentrations of monoclonal antibodies OKM1 or M1/70, both directed against the α subunit of Mac-1, CD11b (23), completely abolished the binding of 125I-D90 to fMLP-stimulated suspensions of PMN (Table I). In contrast, monoclonal antibodies reacting with a different epitope on the α subunit (60.1) or against the β subunit, CD18 (60.3, IB4), did not interfere with 125I-D90 binding to Mac-1 (Table I).

To conclusively establish the structural organization of D90, direct microsequence analysis was carried out. We found that the NH2 termini in the Aα chain remnant of this plasmic fragment originated with residues Leu146, Gln147, Lys148, and Asn149. This α chain origin of D90 thus excludes the NH2-

Fig. 2. A, plasmic fragments of fibrinogen. The inhibitory fibrinogen fragments were analyzed by 10% SDS-gel electrophoresis followed by Coomassie Blue staining. Lane 1 contains aliquots of fibrinogen fragment D migrating under nonreducing conditions with M, ~85,000, in agreement with previous observations (36, 38). After reduction, fragment D appeared as a predominant band at M, ~38,000 and a fainter component at M, ~26,000 (lane 2). Lanes 3 and 4 contain reduced samples of fibrinogen fragment X or intact fibrinogen, respectively. β, reactivity of fibrinogen or fibrinogen fragments with monoclonal antibody 4A5. Increasing concentrations of fibrinogen or of the various fibrinogen fragments (vertical axis) were immobilized on 96-well microtiter plates as described under “Materials and Methods” and assayed for their reactivity with monoclonal antibody 4A5, specific for the COOH-terminal dodecapeptide of the fibrinogen γ chain (31). Absorbance at 405 nm (horizontal axis) measures the binding of monoclonal antibody 4A5 to the various substrates.

Fig. 3. Effects of M, ~30,000 fragment D (D90) (●) or fibrinogen fragment E (●) on 125I-fibrinogen binding to PMN. The experimental procedures are the same as in Fig. 1. The effects of D90 or fragment E on 125I-fibrinogen binding to fMLP-stimulated PMN are shown as mean ± S.E. of three independent experiments. 125I-Fibrinogen bound in the absence of competitors was 99,600 ± 4,500 molecules/cell.

Fig. 4. Binding of 125I-D90 to PMN. Suspensions of PMN were incubated as described in Fig. 1 in the presence of increasing concentrations of 125I-D90 for 20 min at room temperature. Specific binding was calculated in the presence of 100-fold molar excess unlabeled fibrinogen or D90 and was subtracted from the total to calculate specific binding (O). Each point is the mean ± S.E. of three independent experiments. Inset, analysis of 125I-D90 bound to the cell surface by SDS electrophoresis on a 15% polyacrylamide gel under nonreducing (lane 1) or reducing conditions (lane 2).
terminal RGD sequence at residues 95-97 (41), while for its $M_r \sim$ 11,000-13,000 D$_{ab}$ must terminate to omit the COOH-terminal RGD at position 572-574 (41). The origin of D$_{ab}$ was also assigned at residues Arg$^8$, Arg$^9$, Glu$^{10}$, and Glu$^{11}$ in the $\beta$ chain at and residues Met$^{18}$, Leu$^{20}$, Glu$^{21}$, and Glu$^{22}$ in the $\gamma$ chain. Furthermore, as demonstrated above, and in agreement with previous observations (36-38), plasmic digestion of fibrinogen originating the $M_r \sim$ 80,000 fragment D is already associated with proteolytic cleavage and removal of the COOH terminus of the $\gamma$ chain. These findings are difficult to reconcile with a previously reported inhibition of fibrinogen interaction with PMN mediated by synthetic analogues of the COOH terminus of the $\gamma$ chain unless an entirely distinct recognition is involved in Mac-1 binding to immobilized fibrinogen (24).

In addition to the inhibition of $^{125}$I-D$_{ab}$ binding to PMN mediated by intact fibrinogen, we have further substantiated the specificity of this interaction using a panel of monoclonal antibodies. Complete inhibition of $^{125}$I-D$_{ab}$ binding to stimulated PMN was achieved with the same monoclonal antibodies that in previous studies abolished the binding of $^{125}$I-fibrinogen to leukocytes (23) and directed it to the $\alpha$, Mac-1-specific, subunit of this receptor. Interestingly, monoclonal antibodies against the $\beta$ subunit of Mac-1 did not reduce binding of $^{125}$I-D$_{ab}$, leading to the speculation that the complementary fibrinogen interacting site on Mac-1 might reside on its $\alpha$ subunit, in analogy with the preferential platelet $\alpha$IIb-$\beta$3-mediated recognition of fibrinogen (39, 40, 42).

In summary, we have identified a novel interacting region that mediates the assembly of fibrinogen on the leukocyte surface. Integrin receptors homologous to Mac-1 display fibrinogen binding capacity on a variety of cells in a reaction crucially regulated by recognition of the RGD sequence and/or the COOH-terminal dodecapeptide of the $\gamma$ chain (15, 18, 27, 28, 34, 35, 39). The platelet integrin $\alpha$IIb-$\beta$3 well exemplifies the complexity of this simultaneous recognition of two noncontiguous regions in the fibrinogen molecule (40), coordinating a process of central importance in the maintenance of normal hemostasis and vessel wall integrity. On leukocytes, fibrinogen deposition participates in broad mechanisms of inflammation associated with fibrin formation (9-12). The relevance of this process is underscored by its biologic alterations when leukocytes play a major role in the pathophysiology of vascular obstruction and in atherothrombotic disease (5-8). These important functional differences are perhaps reflected in the unique structural recognition of Mac-1 for fibrinogen that does not involve the RGD sequence nor the COOH-terminal dodecapeptide of the $\gamma$ chain. The remarkable affinity of this interaction combined with its rigorous specificity suggest its therapeutic potential as an inhibitor of leukocyte adhesion without interfering with hemostatic functions dependent upon RGD-directed integrins (15, 39).

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