Dual Regulation of Ligand Binding by CD11b I Domain

INHIBITION OF INTERCELLULAR ADHESION AND MONOCYTE PROCOAGULANT ACTIVITY BY A FACTOR X-DERIVED PEPTIDE

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The role of coagulation factor X as a ligand for CD11b/CD18 (Mac-1, aMβ2) in leukocyte adhesion was investigated. A factor X peptide, (G)L238YQAKRFKV(G)246 (peptide 1), blocked ligand binding to CD11b/CD18 and prevented monocyte procoagulant activity. This peptide also inhibited monocytic THP-1 cell adhesion to tumor necrosis factor α-stimulated endothelium and blocked neutrophil migration through tumor necrosis factor α-activated endothelial cell monolayers. In contrast, other factor X-derived peptides were ineffective. Radiolabeled peptide (G)LYQAKRFKV(G) bound specifically and saturably to isolated recombinant CD11b I domain. Functionally, the factor X sequence (G)LYQAKRFKV(G) dose-dependently inhibited THP-1 cell attachment to intercellular adhesion molecule 1 (ICAM-1) transfected cells (IC50 = ~50 μg/ml), indistinguishably from anti-CD18 monoclonal antibodies 60.3 and IB4. In contrast, peptide (G)LYQAKRFKV(G) failed to reduce binding of 125I-fibrinogen to immobilized CD11b I domain, which was abolished by the fibrinogen-derived peptide KYG190WTYQFRKLDGSYG202. By Lineweaver-Burke analysis, peptide (G)LYQAKRFKV(G) inhibited factor X binding to CD11b/CD18 in a noncompetitive fashion, and intact factor X did not reduce monocyte-endothelial cell interaction. These data suggest that the factor X sequence (G)LYQAKRFKV(G) defines an ICAM-1-binding site on CD11b I domain physically distinct from and nonoverlapping with the fibrinogen interacting region(s). Engagement of this site induces a conformational change in the holo receptor, which disrupts a distant factor X-binding site required for monocyte procoagulant activity. These observations demonstrate a dual regulatory role of CD11b I domain in ligand binding and provide a molecular basis for the recently reported anti-inflammatory properties of factor X homologous sequences in vivo.

Leukocyte β2 integrins CD11a-d/CD18 maintain adherence mechanisms (1), transmembrane signaling (2, 3), and intercellular communication (4, 5) in disparate inflammatory responses (5, 6). Specifically, leukocyte adhesion mediated by CD11b/CD18 (Mac-1, aMβ2) (1) depends on the recognition of unrelated ligands, including counter-receptors, i.e. intercellular adhesion molecules (ICAMs) (7, 8), and soluble proteins, like complement C3b (1), fibrinogen and coagulation factor X (9). Blocking monoclonal antibodies (mAbs) (10), direct binding studies to the isolated recombinant protein (11), and mutagenesis experiments (12–14) converged to identify the ~200-aa inserted "I" domain in the α subunit as a recognition site for ICAM-1, fibrinogen, and C3bi. However, how these I domain-binding sites for unrelated ligands are structurally and functionally organized has not been completely elucidated. Although differential inhibition by epitope-mapped mAbs proposed the existence of functionally independent I domain subregions (10), receptor mutagenesis studies suggested that the binding of fibrinogen, C3bi, and ICAM-1 can be mediated, at least in part, by functionally overlapping regions (12, 14, 15).

Differently from other β2 integrin ligands, binding of factor X to CD11b/CD18 (16) is not mediated by the I domain (11), is inhibited by three spatially distant sequences in the ligand (17), and mediates monocyte procoagulant activity (9). In this study, we sought to reinvestigate the association of factor X with CD11b/CD18 and its potential impact on β2 integrin-dependent leukocyte adhesion. Using a small factor X peptide (G)L238YQAKRFKV246 (G) (17), we have delineated a discrete region on CD11b I domain that mediates the interaction with ICAM-1 and indirectly modulates a distant binding site for factor X.

MATERIALS AND METHODS

Cells and Cell Cultures—Polymorphonuclear leukocytes (PMN) were isolated from acid-citrate-dextrose anticoagulated blood drawn from informed normal volunteers by Ficoll-Hypaque gradient density centrifugation and dextran sedimentation (18). Human umbilical vein endothelial cells (HUVECs) were prepared by collagenase treatment and used between passages 2 and 4. The monocyctic cell line THP-1 and CD11b/CD18+ T leukemia cell line MLT (ATCC, Rockville, MD) were maintained in culture according to the manufacturer’s recommendations. THP-1 cell expression of CD11b/CD18 and recognition of fibrinogen and factor X by these cells have been reported (9).

Synthetic Peptides and mAbs—The experimental procedures for the isolation and purification of human plasma fibrinogen have been described previously (9). Aliquots of fibrinogen or factor X were 125I-labeled by the IODO-GEN method to a specific activity of 0.5 and 1–2 Ci/μg of protein, respectively. Integrity and specific activity of 125I-factor X were as described (16). The factor X-derived peptides G238YDTQKED277(G) (peptide 1), G142DSMKTRG250 (peptide 2), and (G)L238YQAKRFKV246 (G) (peptide 16, residues in parentheses added to the natural sequence) were previously characterized for their ability to inhibit 125I-factor X binding to CD11b/CD18 on chemotactic-sim-
peptides before stimulation with 10 μM fMLP (THP-1) and addition to TNFα-stimulated HUVEC for 1 h at 22 °C in the presence of 1 mM CaCl₂. After washes, adherent cells were solubilized in 15% SDS, and radioactivity was determined in a scintillation β counter. Data are means ± S.D. of three independent experiments (p = 0.039). The peptide sequences are: peptide 1, GYDTKQEDG; peptide 2, IDRSMKTRG; peptide 16, GILYQAKRFKV(G); and control peptide 25 (GLEG-FEGKNG). The residues in parentheses were added to the natural sequence.

The effect of factor X peptides on leukocyte-endothelial interaction. 51Cr-labeled THP-1 (A) or CD11b/CD18+ MLT (B) cells (1 × 10⁵) were preincubated with 500 μg/ml of the indicated factor X peptides before stimulation with 10 μM fMLP (THP-1) and addition to TNFα-stimulated HUVEC for 1 h at 22 °C in the presence of 1 mM CaCl₂. After washes, adherent cells were solubilized in 15% SDS, and radioactivity was determined by a one-stage sensitive clotting assay using a factor VII- and factor X-deficient plasma (Sigma) as described (17). 50-min incubation of monocytes or PMN (17). All peptides were synthesized by the W. M. Keck protein chemistry facility at Yale University, purified by high pressure liquid chromatography, and characterized for amino acid composition by mass spectrometry. The factor X peptide (GLLLEGFEKGN²⁵⁶(G) (peptide 25) was used as a control. 2 mg of peptide 16 were iodinated with 5 mCi of Na ¹²⁵I by the IODO-GEN method for 45 min at 4 °C, followed by separation of free from peptide-bound radioactivity by gel filtration over a Bio-Gel P-2 column (Bio-Rad) pre-equilibrated with PBS, pH 7.2. Anti-CD18 mAbs 60.3 and IB4 were obtained from ATCC. Nonbinding mAb 14E11 was used as a negative control.

Expression of Recombinant Proteins—The establishment and characterization of Chinese hamster ovary cells stably transfected with the ICAM-1 cDNA has been reported (19). The construction and expression of recombinant CD11b I domain (residues Gly¹¹¹–Ala³18) and its interaction with fibrinogen and ICAM-1 have been described (11).

Procoagulant Activity and Binding Reactions—The effect of the various factor X peptides on monocyte procoagulant activity was determined by a one-stage sensitive clotting assay using a factor VII- and factor X-deficient plasma (Sigma) as described (17). 50-μl aliquots of purified CD11b I domain at 5 μg/ml in Tris-buffered saline, pH 8.0 were immobilized onto 96-well U-bottom Falcon 3911 flexible assay plates (Becton Dickinson, Oxnard, CA) for 18 h at 4 °C. Duplicate wells were washed in Tris-buffered saline, pH 8.0, post-coated with 10 mg/ml BSA (Sigma) for 90 min at 37 °C, rinsed, and mixed with increasing concentrations of ¹²⁵I-factor X (10–150 μCi/ml) at 14,000 g/ml in PBS, pH 7.2, 2.5 mM CaCl₂ and 0.1% BSA for 30 min at 22 °C. At the end of the incubation, wells were washed twice in PBS, pH 7.2, amputated, and counted in a γ counter. Specific binding was calculated in the presence of a 50-fold molar excess of unlabeled peptide 16 or control peptide 25 added at the start of the incubation. Alternatively, increasing concentrations of ¹²⁵I-fibrinogen (10–150 μg/ml) were incubated with CD11b I domain-coated wells in the presence of 1 mM CaCl₂ and 0.1% BSA for 30 min at 22 °C, before determination of specific binding. For all experiments, nonspecific binding was measured in the presence of a 50-fold molar excess of unlabeled fibrinogen and subtracted from the total to calculate net specific binding. In peptide inhibition experiments, CD11b I domain-coated plates were preincubated with increasing concentrations (0.1–1000 μg/ml) of fibrinogen-derived P1 peptide KYG¹⁰⁶WTVFQKR1DGSV²⁰² (18) or factor X control peptide 25 or peptide 16, preincubated in the presence of 1 mM CaCl₂ and added (1 × 10⁶) to each washed transwell for the indicated time intervals at 37 °C. At the end of the incubation, PMN were recovered from the bottom chamber and counted microscopically. Data are the means ± S.D. of two independent experiments (p < 0.05).

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Leukocyte-Endothelium Interaction—Serum-free suspensions of THP-1 or MLT cells (5 × 10⁶/ml) were labeled with 0.5 mCi of ⁵¹Cr (Na₂CrO₄, du Pont de Nemours, Wilmington, DE) for 2 h at 37 °C with a final incorporation of 0.7–2.1 cpm/cell, washed in PBS, pH 7.4, and suspended in serum-free RPMI 1640. MLT or 10⁻⁵ M fMLP-stimulated THP-1 cells were preincubated with increasing concentrations (60–1000 μg/ml) of factor X peptide 1, 2, or 16 or control peptide 25. After a 30-min incubation at 22 °C, ⁵¹Cr-labeled cells (1 × 10⁶) were added to resting or cytokine-activated (100 units/ml TNFa) for 4 h at 37 °C) HUVEC monolayers for 1 h at 22 °C in the presence of 1 mM CaCl₂. After washes, attached cells were solubilized in 15% SDS, and radioactivity associated under the various conditions was determined in a scintillation β-counter. The number of attached cells was calculated by dividing the cpm harvested by the cpm/cell. In other experiments, ⁵¹Cr-labeled THP-1 cells were preincubated with increasing concentrations of factor X peptides (10–1000 μg/ml) or 25 μg/ml anti CD18 mAbs 60.3 or IB4 or control mAb 14E11 for 30 min at 4 °C before addition to monolayers of ICAM-1 transfectants or wild-type Chinese hamster ovary cells. Data for both panels are expressed as the means ± S.D. of three independent experiments.

RESULTS

Anticoagulant Properties of Factor X Interacting Sequences—Previous data suggested that binding of factor X to CD11b/CD18-expressing monocytes is mediated by three spatially distant sites in the ligand catalytic domain (17). Preincubation of THP-1 cells with a saturating concentration of each of the three

Fig. 3. Effect of factor X peptide (G)LYQAKRFKV(G) on THP-1 cell adhesion to ICAM-1 transfectants. A, serum-free suspensions of ⁵¹Cr-labeled THP-1 cells were preincubated with increasing concentrations of factor X control peptide 25 (Ψ) or peptide 16 (●), mixed with 10 μM fMLP and 1 mM CaCl₂, and added (1 × 10⁶) to monolayers of ICAM-1 transfectants. After a 1-h incubation at 22 °C, wells were washed, and cell adhesion was determined as described in the legend to Fig. 2. B, the experimental conditions are the same as for A, except that ⁵¹Cr-labeled THP-1 cells were incubated with 25 μg/ml anti CD18 mAbs 60.3 or IB4 or control mAb 14E11 for 30 min at 4 °C before addition to monolayers of ICAM-1 transfectants or wild-type Chinese hamster ovary cells. Data for both panels are expressed as the means ± S.D. of three independent experiments.

Leukocyte-Endothelium Interaction—Serum-free suspensions of THP-1 or MLT cells (5 × 10⁶/ml) were labeled with 0.5 mCi of ⁵¹Cr (Na₂CrO₄, du Pont de Nemours, Wilmington, DE) for 2 h at 37 °C with a final incorporation of 0.7–2.1 cpm/cell, washed in PBS, pH 7.4, and suspended in serum-free RPMI 1640. MLT or 10⁻⁵ M fMLP-stimulated THP-1 cells were preincubated with increasing concentrations (60–1000 μg/ml) of factor X peptide 1, 2, or 16 or control peptide 25. After a 30-min incubation at 22 °C, ⁵¹Cr-labeled cells (1 × 10⁶) were added to resting or cytokine-activated (100 units/ml TNFa) for 4 h at 37 °C) HUVEC monolayers for 1 h at 22 °C in the presence of 1 mM CaCl₂. After washes, attached cells were solubilized in 15% SDS, and radioactivity associated under the various conditions was determined in a scintillation β-counter. The number of attached cells was calculated by dividing the cpm harvested by the cpm/cell. In other experiments, ⁵¹Cr-labeled THP-1 cells were preincubated with increasing concentrations of factor X peptides (10–1000 μg/ml) or 25 μg/ml anti CD18 mAbs 60.3 or IB4 or control mAb 14E11 for 30 min at 4 °C before addition to monolayers of ICAM-1 transfectants or wild-type Chinese hamster ovary cells, with determination of cell adhesion after a 1-h incubation at 22 °C. For transendothelial cell migration, HUVEC were grown to confluency onto gelatin-coated porous transwell membranes (8-µm diameter; Costar, Cambridge, MA) and stimulated with 100 units/ml TNFa for 4 h at 37 °C prior to the experiment. PMN (1 × 10⁶) were preincubated with 500 μg/ml factor X peptide 16 or control peptide 25 for 30 min at 22 °C, stimulated with 10 μM fMLP in the presence of 1 mM CaCl₂, and added (1 × 10⁶) to HUVEC for increasing time intervals (30–120 min).

Fig. 4. Binding of ¹²⁵I-peptide (G)LYQAKRFKV(G) to isolated CD11b I domain. Recombinant CD11b I domain was immobilized at 5 μg/ml in Tris-buffered saline, pH 8.0, onto plastic microtiter plates for 18 h at 4 °C. Duplicate wells were incubated with the indicated increasing concentrations of ¹²⁵I-peptide (G)LYQAKRFKV(G) in the presence of 1 mM CaCl₂ and 0.1% BSA for 30 min at 22 °C. Wells were washed twice in PBS, pH 7.2, and radioactivity associated under the various conditions was determined in a γ counter. Specific binding was calculated in the absence (■) or in the presence of a 50-fold molar excess of unlabeled factor X control peptide 25 GLEGFEKGN (▲) or peptide 16 (GLYQAKRFKV(G)) added at the start of the incubation. Data are expressed as the means ± S.D. of two independent experiments.

Fig. 5. Effect of factor X- or fibrinogen-derived peptides on ¹²⁵I-fibrinogen binding to CD11b I domain. The experimental conditions are the same as in Fig. 4, except that immobilized CD11b I domain was preincubated with the indicated increasing concentrations of fibrinogen chain F1 peptide KYGKWVYRQKRLGDGSV (▲), factor X control peptide 25 GLEGFEKGN (●), or peptide 16 (GLYQAKRFKV(G)) (■) for 30 min at 22 °C. Wells were incubated with 25 μg/ml ¹²⁵I-labeled fibrinogen in the presence of 1 mM CaCl₂ and 0.1% BSA for 30 min at 22 °C, washed, amputated, and counted in a γ counter, and specific binding was determined. Data are expressed as the means ± S.D. of two independent experiments.

at 37 °C. At the end of each incubation, migrated PMN were recovered from the bottom well, washed, stained with 0.2% trypan blue, and counted microscopically. Before each experiment, the integrity of the endothelial cell monolayer was confirmed by methyl green staining and fluorescence microscopy.

RESULTS

Anticoagulant Properties of Factor X Interacting Sequences—Previous data suggested that binding of factor X to CD11b/CD18-expressing monocytes is mediated by three spatially distant sites in the ligand catalytic domain (17). Preincubation of THP-1 cells with a saturating concentration of each of the three
implicated sequences inhibited factor X-dependent monocyte procoagulant activity by 60–70% in a time-dependent reaction (not shown) and in agreement with previous observations (17, 20).

Effect of Factor X Peptides on Leukocyte-Endothelium Interaction—Preincubation of monocytic THP-1 cells with an inhibitory concentration (500 μM) (17) of factor X peptide 16 (G)L728YQAKRFKV240(G) significantly inhibited monocyte attachment to TNFα-activated HUVEC from 67 ± 7.5 to 31 ± 6.6% (Fig. 1A). In contrast, the factor X peptides G366YDTKQED373(G) (peptide 1) or I422DRSMKTRG430 (peptide 2), which have been demonstrated (17) to block ligand binding to CD11b/CD18 or control factor X peptide (G)L72EGFEGK(G) (peptide 25), did not reduce THP-1 cell adhesion to HUVEC (Fig. 1A). At variance with the effect on monocyte cell adhesion, none of the factor X peptides, including peptide 16, decreased attachment of CD11b/CD18+ T cells (MLT) to TNFα-activated endothelium under the same experimental conditions (Fig. 1B). Inhibition of monocytic cell adhesion to HUVEC by the factor X peptide (G)L72YQAKRFKV(G) was specific and dose-dependent (Fig. 2A), whereas control peptide 25 was ineffective (Fig. 2A). In other experiments, the factor X peptide 16 specifically inhibited fMLP-stimulated PMN transendothelial cell migration at all time intervals tested (Fig. 2B).

Effect of Factor X Peptide 16 on CD11b I Domain Ligand Recognition—The possibility that the factor X peptide 16 may affect intercellular adhesion mediated by CD11b/CD18–ICAM-1 interaction was first investigated. Increasing concentrations of peptide 16 blocked the adhesion of fMLP-stimulated THP-1 cells to monolayers of ICAM-1 transfectants in a concentration-dependent manner, with an IC50 value of 4.32 μM (Fig. 5), in agreement with previous observations (17). However, analysis of binding data by the Lineweaver-Burke plot revealed that only the factor X sequence (G)DRSMKTRG inhibited ligand binding in a genuine competitive manner, as judged by the nearly identical y intercept values determined in the presence (y = 4.32) or in the absence (y = 4.32) of antagonist (Fig. 6). In contrast, the factor X sequences GYDTKQEDG and (G)L72YQAKRFKV(G) inhibited factor X binding to CD11b/CD18 in noncompetitive manner, with 4–5-fold differences in the predicted y intercept values in control curves (y = 4.32) or in the presence (y = 21.7; Ref. 18) of these inhibitors (Fig. 6).

DISCUSSION

In this study, we have shown that a factor X sequence, (G)L72YQAKRFKV240(G), exerted a dual anticoagulant and anti-inflammatory effect by blocking monocyte procoagulant activity and inhibiting leukocyte-endothelium interaction. This pathway involved peptide engagement of a discrete ICAM-1-binding site on CD11b I domain with indirect disruption of a distant factor X-binding region in the holoreceptor.

Through its promiscuous ligand repertoire (2), CD11b/CD18 mediates a variety of cell adhesive interactions and functions as a procoagulant receptor via its high affinity recognition of factor X (9). The possibility that this interaction may also participate in leukocyte adhesion mechanisms has been postulated. Previously, interruption of CD11b/CD18-dependent coagulation prevented thrombin-dependent chemotaxis (21) and monocyte adhesion (22, 23) to virally infected endothelium (17). Moreover, an adhesive determinant of Bordetella pertussis, filamentous hemagglutinin, was recently shown to contain sequences homologous to the three factor X regions inhibiting ligand binding to CD11b/CD18 (20). Synthetic peptidyl mimetics of these regions produced a potent anti-adhesive and anti-inflammatory effect by blocking leukocyte-endothelium interaction in vitro and reducing neutrophil accumulation in the colony-stimulating factor in an in vivo model of bacterial meningitis (20). However, the molecular basis of this anti-inflammatory pathway was not elucidated.

Here, at variance with the anti-adhesive functions of all three factor X homologous sequences (20), a single factor X
peptide (G)L238YQARKFKV246(G) (peptide 16) acted as a potent antagonist of leukocyte-endothelium interaction and transendothelial cell migration. Three independent lines of evidence indicate that this anti-adhesive effect resulted from peptide engagement of an ICAM-1-binding site on CD11b I domain (10–13). First, this peptide had no effect on CD11b/CD18− T cell (MLT) adhesion to endothelium. Second, binding of monocyte THP-1 cells to monolayers of ICAM-1 transfectants was equally well inhibited by factor X peptide 16 or by anti-CD18 mAbs. Thirdly, direct radiolabeled peptide binding studies demonstrated that this sequence physically interacted with CD11b I domain. The use of a small synthetic peptide, like peptide 16, to probe the CD11b/CD18 ligand repertoire allowed delineating a more precise functional map of CD11b I domain.

Consistent with the differential pattern of inhibition obtained with I domain epitope-mapped mAbs (10), the ability of this peptide to disrupt an ICAM-1-binding site without affecting the recognition of fibrinogen postulates the existence of physically separate subdomains mediating a nonoverlapping recognition of these two ligands. This is at variance with previous studies of site-directed mutagenesis, in which single amino acid changes in the CD11b metal ion-dependent adhesion site (15) suppressed the receptor recognition of fibrinogen, ICAM-1, and C3bi (12, 14, 15). This may be explained by the ability of the metal ion-dependent adhesion site to transduce conformational effects in spatially distant ligand-binding sites (see below) without affecting subunit assembly or receptor surface expression.

A potential model of how perturbation of a discrete I domain region, i.e. ICAM-1 site, could indirectly modulate ligand binding to the holoreceptor was provided by dissecting the factor X interaction with CD11b/CD1. Although three spatially distant sites in factor X indistinguishably blocked ligand binding and monocyte procoagulant activity (17), only one of these regions, 1422DRSMKTRG430, was a genuine competitive inhibitor of this interaction. The other two factor X peptides, including peptide 16, were noncompetitive antagonists, thus potentially disrupting a secondary docking during receptor-ligand interaction. This suggests that binding of the anti-adhesive sequence (G)L1422YQARKFKV(G) to an ICAM-1 recognition site on CD11b I domain may induce a conformational change in the holoreceptor with disruption of a distant factor X-binding pocket. Consistent with this hypothesis, 125I-factor X did not specifically associate with recombinant isolated CD11b I domain (11), and in the present studies, the intact macromolecule did not affect leukocyte-endothelium interaction. Altogether, these data suggest a more dynamic role of β2 integrin I domains, not only in providing physical interacting site(s) for ligand recognition, but also in regulating the state of receptor activation (24) and ligand binding affinity (25).

In summary, these data underscore the role of the CD11b I domain in multiple regulation of ligand binding and identify a factor X sequence that, similarly to its prokaryotic homologue (20), displayed potent anticoagulant and anti-inflammatory properties. The availability of a small and high affinity probe like peptide 16 should facilitate the identification of the complementary ICAM-1-binding site on CD11b and define its role in leukocyte traffic and recirculation (4, 5).

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