Activation-dependent Exposure of the Inter-EGF Sequence Leu$^{83}$-Leu$^{88}$ in Factor Xa Mediates Ligand Binding to Effector Cell Protease Receptor-1*  

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Binding of factor Xa to human umbilical vein endothelial cells (HUVEC) is contributed by effector cell protease receptor-1 (EPR-1). The structural requirements of this recognition were investigated. Factor Xa or catalytically inactive 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) Glu-Gly-Arg-(DEGR)-chloromethylketone-factor Xa bound indistinguishably to HUVEC and EPR-1 transfectants, and inhibited equally well the binding of $^{125}$I-factor Xa to these cells. Similarly, factor Xa active site inhibitors TAP or NAP5 did not reduce ligand binding to EPR-1. A factor X peptide duplicating the inter-EGF sequence Leu$^{83}$-Phe$^{84}$-Thr$^{85}$-Arg$^{86}$-Lys$^{87}$-Leu$^{88}$-(Gly) inhibited factor V/Va-independent prothrombin activation by HUVEC and blocked binding of $^{125}$I-factor Xa to these cells in a dose-dependent manner (IC$_{50}$ ~ 20–40 μM). In contrast, none of the other factor X peptides tested or a control peptide with the inter-EGF sequence in scrambled order was effective. A recombinant chimeric molecule expressing the factor X sequence Leu$^{83}$-Leu$^{88}$ within a factor IX backbone inhibited binding of $^{125}$I-factor Xa to HUVEC and EPR-1 transfectants in a dose-dependent fashion, while recombinant factor IX or plasma IXa had no effect. An antibody generated against the factor X peptide 83–88, and designated JC15, inhibited $^{125}$I-factor Xa binding to HUVEC. The JC15 antibody bound to factor Xa and the recombinant IX/X83–88 chimera in a concentration dependent manner, while no specific reactivity with factors X or IXa was observed. Furthermore, binding of $^{125}$I-factor Xa to immobilized JC15 was inhibited by molar excess of unlabeled factor Xa, but not by comparable concentrations of factors X or IXa. These findings identify the inter-EGF sequence Leu$^{83}$-Leu$^{88}$ in factor Xa as a novel recognition site for EPR-1, and suggest its potential role as a protease activation-dependent neo-epitope. This interacting motif may help elucidate the contribution of factor Xa to cellular assembly of coagulation and vascular injury.

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Among vascular cells, monocytes and endothelial cells contribute to hemostasis by regulating the assembly of clotting and fibrinolytic proteases (1). In addition to negatively charged phospholipids (1), this process is contributed by a variety of structurally and evolutionarily unrelated cell surface receptors. These include receptors for anticoagulant protein C/activated protein C (2), fibrinolytic protein urokinase (3), and coagulation zymogens/proteases thrombin (4), factors VIIa (5), XII (6), IX/Xa (7), X (8), and Xa (9). Protease receptors are also potent signaling molecules, regulating the generation of second messengers (10, 11), gene transcription and cytokine release (12, 13), cell proliferation (6, 14–16), and inflammatory (17) or anti-inflammatory responses (18).

Effect receptor cell protease receptor-1 (EPR-1)$^1$ functions as a receptor for factor Xa on leukocytes (9) and endothelial cells (19), thus enhancing factor V/Va-independent prothrombin activation and leukocyte costimulation (17). On activated platelets, EPR-1-factor Xa interaction contributes to membrane assembly of the prothrombinase complex (20). For the procoagulant potential of factor Xa in vivo (21) and its mitogenic activity on endothelial and smooth muscle cells (15, 16), factor Xa-cellular interactions may directly contribute to the pathogenesis of vascular injury (22).

In this study, we sought to investigate the structure-function requirements of EPR-1-factor Xa interaction and the receptor specificity for the active protease versus the zymogen factor X. Using synthetic peptides, a recombinant factor IXN chimera, and a sequence-specific antibody, we found that the interconnecting EGF sequence Leu$^{83}$-Phe$^{84}$-Thr$^{85}$-Arg$^{86}$-Lys$^{87}$-Leu$^{88}$-(Gly) in factor Xa mediates ligand binding to EPR-1 and becomes surface exposed only after zymogen activation.

**EXPERIMENTAL PROCEDURES**

**Proteins and Protein Labeling**—The experimental procedures for the isolation and characterization of human plasma factors IX and X and the generation of the corresponding active proteases IXa and Xa have been reported (8). Aliquots of factor Xa purchased from Calbiochem or Haematologic Technologies Inc. (Essex Junction, VT) gave indistinguishable results in binding assays and thrombin generation experiments. Dansyl-Glu-Gly-Arg (DEGR)-chloromethylketone factor Xa and human prothrombin were purchased from Haematologic Technologies and Calbiochem, respectively. Factor Xa active site inhibitors TAP and NAP5 (23), and NAPe2, which recognizes a factor X exosite involved in zymogen activation by tissue factor-factor VIIa, were generously provided by Dr. G. Vlasuk (Corvas International, San Diego, CA). A library of factor X peptides, including the inter-EGF sequence Leu$^{83}$-Phe$^{84}$-Thr$^{85}$-Arg$^{86}$-Lys$^{87}$-Leu$^{88}$-(Gly) and its scrambled variant Lys-

1 The abbreviations used are: EPR-1, effector cell protease receptor-1; CHO, Chinese hamster ovary; HUVEC, human umbilical vein endothelial cells; DMEM, Dulbecco's modified Eagle's medium; TBS, Tris-buffered saline; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; TAP, tick anticoagulant peptide; NAP, nematode anticoagulant peptide.
Phe-Thr-(Gly)-Arg-Leu-Leu (residues in parentheses added to the natural sequence), was synthesized and characterized previously (24). Factor X numbering was according to Fung et al. (25). Aliquots (2.1–5.4 μl) of factor Xa or DEGR-factor Xa were radiolabeled with 125I-NaI (Amersham Corp.) by the IODO-GEN method (26) to a specific activity of 0.4–1.5 mCi/μg. Supernatants were separated from unbound radiolabeled factor Xa by gel filtration on a Sephadex G-25 column pre-equilibrated with phosphate buffered saline, pH 7.4, and collection of 0.5 ml fractions. Both unlabeled factor Xa and 125I-factor Xa indistinguishably cleaved the factor Xa-sensitive chromogenic substrate S-2222.

Construction of a Recombinant Factor IX/X83–88 Chimera—A recombinant factor IX/X83–88 chimera was synthesized and characterized previously (24). Aliquots (2.1–5.4 μl) of factor Xa or DEGR-factor Xa were radiolabeled with 125I-NaI (Amersham Corp.) by the IODO-GEN method (26) to a specific activity of 0.4–1.5 mCi/μg. Supernatants were separated from unbound radiolabeled factor Xa by gel filtration on a Sephadex G-25 column pre-equilibrated with phosphate buffered saline, pH 7.4, and collection of 0.5 ml fractions. Both unlabeled factor Xa and 125I-factor Xa indistinguishably cleaved the factor Xa-sensitive chromogenic substrate S-2222.

Binding Studies—HVEUC were isolated by collagenase treatment and maintained in DMEM tissue culture medium (BioWhittaker), supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and endothelial cell growth factor, plated onto gelatinized 48-well tissue culture plates (Costar Corp., Cambridge, MA) at a density of 4 × 104 cells/well, and cultured for 2–4 d prior to the assay. Cells were washed twice with serum-free RPMI 1640 and incubated in a total volume of 300 μl with 2.5 nM 125I-factor Xa or 10.8 nM factor Xa for 10 min in the absence of increasing concentrations (0.1–1 μl) of unlabeled competitors, factors X, IXa, or DEGR-Xa. For competition experiments with recombinant factor IX or the IX/X83–88 chimera, 5.43 nM 125I-factor Xa was used. After a 15-min incubation at 22°C, cells were washed three times in serum-free medium and solubilized in 10% SDS, and radioactivity associated with the cell monolayer under the various conditions tested was determined in a γ counter. In parallel experiments, CHO cells transiently transfected with the EPR-1 DNA (29) were detached by phosphate-buffered saline-EDTA, pH 7.4, washed, diluted to 2.0 × 105 cells/ml in serum-free medium and solubilized in 10% SDS, and radioactivity associated with the cell monolayer under the various conditions tested was determined in a γ counter. In parallel experiments, CHO cells transiently transfected with the EPR-1 DNA (29) were detached by phosphate-buffered saline-EDTA, pH 7.4, washed, diluted to 2.0 × 105 cells/ml in serum-free medium and solubilized in 10% SDS, and radioactivity associated with the cell monolayer under the various conditions tested was determined in a γ counter. In parallel experiments, CHO cells transiently transfected with the EPR-1 DNA (29) were detached by phosphate-buffered saline-EDTA, pH 7.4, washed, diluted to 2.0 × 105 cells/ml in serum-free medium and solubilized in 10% SDS, and radioactivity associated with the cell monolayer under the various conditions tested was determined in a γ counter.
experiments is nonsaturating for the system, and has been used for comparison with previous data obtained with other EPR-1- (9). No specific thrombin generation was detected in the absence of cells, under the same experimental conditions (not shown).

Production and Characterization of Sequence-specific JC15 Antibody-A sequence-specific antibody was generated in a rabbit by multiple subcutaneous injections in complete Freund’s adjuvant of 100 μg of the inter-EGF factor X peptide LFTRKL(G) preparatively coupled to keyhole limpet hemocyanin. After a 4-week interval, animals were boosted with subcutaneous injection of 100 μg of peptide in incomplete Freund’s adjuvant and sequentially boosted and bled at alternate weeks. Rabbit immunoglobulin fractions of the relevant serum, designated JC15, were purified by affinity chromatography on protein A-Sepharose and used for inhibition of 125I-factor Xa binding to EPR-1-expressing cells, as described above. The recognition specificity of JC15 antibody was characterized by enzyme-linked immunosorbent assay. Ninety-six well plastic microtiter plates (Costar Corp., Cambridge, MA) were coated with 0.21 μg/ml factors Xa, X, or Xa, or the IXXX53–88 chimera in Tris-buffered saline (TBS), pH 7.4, in a total volume of 100 μl for 18 h at 4 °C. After washes in TBS, pH 7.4, wells were post-coated with 3% gelatin (Sigma) in TBS, pH 7.4, for 60 min at 37 °C, and mixed with increasing concentrations (1.25–50 μg/ml) of control non immune rabbit IgG or JC15 antibody in TBS, pH 7.4, containing 0.05% Tween-20 plus 1% bovine serum albumin (Sigma) for 90 min at 37 °C. After washes, binding of the primary antibody was revealed by addition of a 1:4000 dilution of alkaline phosphatase-conjugated streptavidin reagent (Zymed) for 1 h washes, binding of the primary antibody was revealed by addition of a 1:4000 dilution of biotin-conjugated goat anti-rabbit IgG (Zymed) for 1 h washes, binding of the primary antibody was revealed by addition of a 1:4000 dilution of alkaline phosphatase-conjugated streptavidin reagent (Zymed) and p-nitrophenyl phosphate for 30 min at 37 °C, with determination of absorbance at A405.

RESULTS

Catalytic Active Site-independent Binding of Factor Xa to EPR-1—Previous studies demonstrated that EPR-1 bound factor Xa but not the zymogen factor X (9). A potential requirement of factor X proteolytic activity in ligand binding to EPR-1 was investigated. Factor Xa active site inhibitors TAP, NAP5 (23), or NAPc2, did not reduce 125I-factor Xa binding to HUVEC (Table I). Similar to catalytically inactive DEGR-factor Xa binding of 125I-factor Xa to HUVEC (19) in a concentration-dependent reaction, quantitatively indistinguishable from that observed with the active protease (Fig. 1A). Consistent with these data, 125I-DEGR-factor Xa bound specifically to HUVEC, in a reaction inhibited equally well by increasing molar excess of factor Xa or DEGR-factor Xa (Fig. 1B). In control experiments, TAP- or NAP5-treated factor Xa, or DEGR-factor Xa, were completely devoid of catalytic activity by S-2222 hydrolysis (not shown).

Synthetic Peptidyl Mimicry of the EPR-1 Binding Site on Factor Xa—Screening of factor X sequences (24) revealed that a synthetic peptide corresponding to the inter-EGF region Leu103-Phe104-Thr105-Arg106-Lys107-Leu108-(Gly) inhibited HUVEC prothrombin activation in the absence of factor V/Va by 10% (Fig. 2). With the exception of the factor X sequence 366–375, which produced only a partial and variable reduction in HUVEC prothrombin activation, none of the other factor X peptides tested, including antagonists of factor X binding to Mac-1 (24), were effective under the same experimental conditions (Fig. 2).

| Inhibitor | HUVEC | EPR-1 transfectants |
|-----------|-------|---------------------|
| None      | 0.22 ± 0.02 | 1.2 ± 0.01 |
| TAP       | 0.34 ± 0.06 | 1.1 ± 0.03 |
| NAP5      | 0.27 ± 0.07 | 1.4 ± 0.13 |
| NAPc2     | 0.17 ± 0.04 | 1.4 ± 0.22 |

Table I

Effect of factor Xa inhibitors on 125I-factor Xa binding to EPR-1-expressing cells

Aliquots (10.8 nM) of 125I-factor Xa were incubated with 100 nM concentrations of the indicated factor Xa inhibitors for 20 min at 22 °C, before addition to HUVEC monolayers or suspensions of CHO cells transiently transfected with the EPR-1 cDNA in a total volume of 300 μl for an additional 20-min incubation at 22 °C. Specific binding for both cell types was calculated as described under “Experimental Procedures.” Data are the mean ± S.E. of at least three independent experiments.

Fig. 1. Catalytic active site-independent binding of factor Xa to EPR-1. HUVEC were equilibrated in a total volume of 300 μl with 10.8 nM 125I-factor Xa (A) or 125I-DEGR-factor Xa (B) and 2.5 mM CaCl2, in the presence or in the absence of the indicated increasing concentrations (0.1–1 μM) of unlabeled factor Xa (●) or DEGR-factor Xa (○). After a 15 min incubation at 22 °C, cells were washed three times in serum-free RPMI 1640, solubilized in 10% SDS, and cell-associated radioactivity was determined in a γ counter. One hundred percent specific binding was 0.56 ± 0.13 nM (A) or 0.73 ± 0.1 (B). Data are the mean ± S.E. of at least three independent experiments.

Con-
consistent with the requirement of EPR-1-factor Xa interaction for prothrombin activation (29), binding of $^{125}$I-factor Xa to HUVEC was inhibited in a dose-dependent manner by the factor X peptide 83–88, but not by the 83–88 control scrambled peptide, nor by the COOH-terminus EGF peptide 101–108 (Fig. 3B). The higher peptide concentrations required to inhibit factor Xa-HUVEC interaction as compared with prothrombin activation (Fig. 3) suggests heterogeneity in ligand binding, potentially comprising both functional (EPR-1-mediated) and nonfunctional cellular associations. In control experiments, increasing concentrations (1–200 nM) of factor X peptides 83–88 or 101–108 did not reduce factor Xa- or thrombin-dependent hydrolysis of S-2222 and S-2238, respectively, ruling out a potential substrate competition mechanism for inhibition of cell prothrombin activation (not shown).

Molecular Characterization of the EPR-1 Ligand Binding Site—A recombinant chimeric molecule containing the factor X inter-EGF sequence Leu83-Leu88 in factor Xa was engineered into the framework of factor IX, purified by monoclonal antibody affinity chromatography, and tested in gain-of-function experiments for inhibition of $^{125}$I-factor Xa binding to EPR-1-expressing cells. Gla analysis of the factor IX/X83–88 chimera showed a Gla content similar to that of control plasma-derived protein (9.1 mol of Gla/mol of protein for the recombinant chimeric protein and 7.9 mol of Gla/mol of protein for plasma-derived factor X). Under these experimental conditions, binding of $^{125}$I-factor Xa to HUVEC (Fig. 4A) or EPR-1 transfectants (Fig. 4B) was dose-dependently inhibited by increasing molar excess of unlabeled factor Xa or thrombin-dependent hydrolysis of S-2222 and S-2238, respectively, ruling out a potential substrate competition mechanism for inhibition of cell prothrombin activation (not shown).

In this study, we have shown that the interconnecting EGF sequence Leu83-Leu88 in factor Xa mediates ligand binding to HUVEC, while incubation of factor Xa with comparable concentrations of control non immune rabbit IgG was without effect (Fig. 5).

Activation-dependent Exposure of the EPR-1 Ligand Binding Site—Potential changes in accessibility of the inter-EGF sequence 83–88 during zymogen activation were investigated. First, in enzyme-linked immunosorbent assay, JC15 antibody bound to immobilized factor Xa or the recombinant IX/X83–88 chimera in a concentration-dependent fashion, while no specific reactivity with factor IXa or the zymogen factor X was observed, under the same experimental conditions (Fig. 6A). In contrast, comparable concentrations of unlabeled recombinant factor IX, or plasma-derived IXa, did not decrease binding of $^{125}$I-factor Xa to EPR-1-expressing cells (Fig. 4A). In another series of experiments, a sequence-specific antibody, designated JC15, was generated against the factor X peptide 83–88 and tested for inhibition of EPR-1-factor Xa interaction. As shown in Fig. 5, preincubation of $^{125}$I-factor Xa with JC15 antibody resulted in dose-dependent inhibition of ligand binding to immobilized factor Xa or the zymogen factor X was observed, under the same experimental conditions (Fig. 6A). In control experiments, comparable concentrations of non immune rabbit IgG did not bind to immobilized factor Xa (Fig. 6A). Second, binding of $^{125}$I-factor Xa to immobilized JC15 was competitively inhibited by molar excess of factor Xa in a concentration-dependent manner (Fig. 6B), while comparable doses of factor IXa or the zymogen factor X did not significantly reduce $^{125}$I-factor Xa binding to JC15-coated plates (Fig. 6B).

**DISCUSSION**

In this study, we have shown that the interconnecting EGF sequence Leu83-Leu88 in factor Xa mediates ligand binding to...
EPR-1. This conclusion was based on synthetic peptidyl mimetic, gain-of-function of a recombinant factor IX/X chimera, and neutralization experiments with a sequence-specific antibody. Moreover, EPR-1 ligand binding could be recapitulated by catalytically inactive factor Xa, thus ruling out a potential requirement of local proteolysis for receptor recognition. Finally, competition studies with the sequence-specific JC15 antibody suggested that the inter-EGF 83–88 region was not accessible in the zymogen factor X, but became surface-exposed in the active protease.

Binding of factor Xa to EPR-1 is one of several receptor-mediated associations between coagulation/fibrinolytic proteases and vascular cells (2–4, 6, 33, 34). In addition to the paradigm of protease-activated receptors (4, 33), these interactions can also be mediated by specific structural requirements, as exemplified by the receptor-binding sequences in the EGF-like modules of urokinase (35), and factor XII (6). Our observations propose an unexpected role for the short inter-EGF sequence 83–88 in mediating docking of factor Xa to EPR-1 and imparting specificity for ligand binding, at least with two different mechanisms. First, the unique structural features of this inter-EGF region, with two unique charged residues Lys86 and Arg87, and its high degree of flexibility (36), as opposed to its "locked" conformation in factor IXa (37), could determine the ability of EPR-1 to bind factor Xa, but not an homologous protease, i.e. factor IXa. Secondly, the accessibility of this interacting motif in factor Xa but not in factor X could dictate the selective recognition of EPR-1 for the active protease, and not for the zymogen factor X. The idea that zymogen activation may result in conformational changes with exposure of selective neo-antigenic epitopes has been proposed earlier. Keyt et al. (38) proposed that factor X activation resulted in conformational transitions in the heavy chain, as well as metal ion-dependent transitions in the heavy and light chains. Conformational remodeling of the protease domain upon factor X activation was also demonstrated by Persson et al. (39) using domain-specific F(ab)2 and factor X-derived tryptic fragments. Similar observations were reported for factors IXa and X upon Ca2+ binding to the Gla module (40, 41). Our competition experiments with the JC15 antibody provide evidence for an additional activation-dependent conformational transition in the light chain of factor Xa, specifically targeted to the inter-EGF 83–88 sequence. Alternatively, this domain may become physically unmasked by the release of the factor X activation peptide during zymogen activation (1). Interestingly, the factor IX/X83–88 chimera inhibited ligand binding to EPR-1 without requiring zymogen activation. The ability of the JC15 antibody to recognize this recombinant protein indistinguishably from native factor Xa suggested that the chimeric insertion produced per se conformational changes and surface exposure of the inter-EGF region.

Current experimental evidence demonstrates that protease receptors initiate multiple cellular signaling pathways. Factor Xa, in particular, has been implicated in endothelial, vascular smooth muscle cell, and leukocyte activation and proliferation mechanisms (15–17), of potential relevance for the pathogenesis of atherosclerosis and vascular diseases (22). Although the data presented here identify the inter-EGF sequence in factor Xa as mediating ligand binding to HUVEC, additional requirements may be involved in post-receptor occupancy events of
factor Xa-dependent signal transduction. In this context, catalytic inactivation of factor Xa abolished EPR-1-stimulated proliferation of HIVUEC and smooth muscle cells (19), and modulation of endothelial cell gene expression.2 Altogether, these data suggest a cooperative model of factor Xa binding to vascular cell EPR-1, potentially involving an initial Gla-dependent contact stabilized by high affinity recognition of the inter-EGF sequence 83–88, and followed by an as yet unidentified step of local proteolysis by cell surface-bound factor Xa for downstream signal transduction events and effector responses (19).

In summary, we have identified the inter-EGF peptide in factor Xa as a novel, activation-dependent sequence for receptor-mediated assembly of coagulation on vascular cells. Antagonists with similar specificity may be beneficial at targeting factor Xa-dependent cellular effector functions in vascular injury and atherosclerosis (21, 22).

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