Mediation of Fibrin-induced Release of von Willebrand Factor from Cultured Endothelial Cells by the Fibrin β Chain

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Abstract

The exposure of endothelial cells (EC) to fibrin has been shown to stimulate the rapid release of von Willebrand factor (vWf) from storage sites in Weibel-Palade bodies. We have now investigated the fibrin structural features required for stimulation of release. The role of fibrinopeptide cleavage was examined by preparing fibrin with thrombin to remove both fibrinopeptide A (FPA) and fibrinopeptide B (FPB) and with reptilase or Agkistrodon contortrix procoagulant to selectively remove FPA or FPB, respectively. vWf release was found to require FPB cleavage, whereas removal of FPA and Factor XIII, cross-linking of fibrin were without effect. The dependence of release on FPB cleavage suggested that a site involving the NH₂ terminus of the β chain could mediate vWf secretion. To test this hypothesis, $B\beta$ chain derivatives were prepared and examined for their capacity to induce release. Purified $B\beta$ chain had no effect on release at a concentration of 20 nM but stimulated release from 26±6% of cells at 200 nM, the maximum solubility. However, after thrombin cleavage of FPB, release occurred from 36±9% of cells at 20 nM and from 60±7% at 200 nM, both significantly greater than before cleavage. FPB and B β 1-42 showed no activity, whereas β 15-42, representing the NH₂ terminus of the thrombin cleaved β chain, stimulated significant release at concentrations of 0.1 and 1 mM. We conclude that FPB cleavage from fibrin is required for stimulation of vWf release from EC and that this is mediated by a site that includes the NH₂ terminus of the β chain.

Introduction

Fibrin interacts with endothelial cells at sites of inflammation, vascular injury, and thrombosis, resulting in several effects on hemostasis and endothelial cell (EC)¹ function. Exposure of a confluent endothelial monolayer to fibrin causes cell retrac-

tion, disruption of cellular organization, and separation of the monolayer into migratory cells (1). Both fibrin and fibrin degradation products have been shown to stimulate EC migration (1-3) and disorganization during angiogenesis (4-6). EC exposed to fibrin increase secretion of both prostacyclin, a potent vasodilator and inhibitor of platelet aggregation, and tissue plasminogen activator, an inducer of fibrinolytic activity (7).

Fibrin may also affect hemostasis by causing release of vWf from its storage sites in the Weibel-Palade bodies of EC (8). vWf is a large adhesive glycoprotein synthesized by EC (9) and megakaryocytes (10, 11) that is required for adhesion of platelets to the subendothelium at sites of vascular injury (12, 13) and also supports the platelet interactions resulting in formation of platelet aggregates (14, 15). The importance of vWf in hemostasis is reflected in the occurrence of the bleeding disorder, von Willebrand's disease, resulting from deficient or defective vWf (16). vWf is structurally heterogeneous, consisting of disulfide-bound multimers varying in molecular weight from 500,000 to 20,000,000 as shown by SDS agarose gel electrophoresis (17, 18), with the larger forms having greater biological activity than the smaller (19). Because vWf stored in EC Weibel-Palade bodies consists of only the largest multimers (20, 21), fibrin-induced secretion can provide highly functional vWf to facilitate local hemostasis.

Fibrinogen is a bivalent molecule composed of six polypeptide chains, two each of the $A\alpha$, $B\beta$, and γ chains. These chains are disulfide bonded near their amino termini, to form a central domain with the carboxyl ends of the chains extending laterally, forming two lateral domains (22). Thrombin cleaves fibrinogen near the amino ends of the $A\alpha$ and $B\beta$ chains liberating fibrinopeptide A (FPA) and fibrinopeptide B (FPB) (23, 24), and generating fibrin monomers that polymerize to form an insoluble gel. Fibrin may be further stabilized by the Factor XIII_a-catalyzed formation of intermolecular ϵ -(γ -glutamyl) lysine isopeptide bonds between γ chains of adjacent monomers (25) and also among two or more α chains (26, 27). Stabilized fibrin forms the supporting matrix of a hemostatic plug or thrombus and is proteolytically degraded by the fibrinolytic system.

Our previous studies (8) indicated that vWf release was stimulated within 10 min of exposure of EC to polymerized fibrin. Specific structural features of fibrin were required for release because fibrin made with reptilase, a snake venom that cleaves only FPA and produces non-cross-linked fibrin (28), was nonstimulatory. In this report, we have investigated the structural features of fibrin and fibrin fragments necessary to support vWf release from EC.

Methods

Fibrinogen and fibrinogen-cleaving enzymes. Lyophilized human fibrinogen (Grade L) was obtained from Helena Laboratories (Beaumont, TX), and copurifying Factor XIII was inactivated with acid-urea treatment as described elsewhere (27). Factor XIII inhibition was con-

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^{1.} Abbreviations used in this paper: EC, endothelial cell; FPA, fibrinopeptide A; FPB, fibrinopeptide B; TFA, trifluoracetic acid.

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firmed by demonstrating absence of cross-linked γ chains by SDS-PAGE of the reduced polypeptide chains of fibrin prepared after clotting by thrombin in the presence of 10 mM calcium chloride. Human thrombin (3,250 U/mg), which cleaves both FPA and FPB, and reptilase snake venom, which cleaves only FPA, were obtained from Calbiochem-Behring Corp. (La Jolla, CA) and Abbott Laboratories (North Chicago, IL), respectively. The FPB cleaving procoagulant fraction of the snake venom from Agkistrodon contortrix (Sigma Chemicals Co., St. Louis, MO) was partially purified by DEAE cellulose chromatography as described elsewhere (29). Procoagulant fractions having no fibrinolytic activity were identified as those that yielded a firm clot after a 3-h incubation at 37°C with fibrinogen (2.3 mg/ml) and that did not lyse with continued incubation for 18 h. These column fractions were pooled and stored at -20° C. The protein concentrations in pooled fractions were determined to be 90 µg/ml using the Bradford method (30).

Preparation of clots. Non-cross-linked fibrin was prepared from 3 mg/ml of Factor XIII-deficient fibrinogen in HBSS with 10 mM calcium chloride and 5×10^{-3} U/ml thrombin, 1.1 batroboxin unit/ml of reptilase or 40 μ g/ml of procoagulant column pool of the contortrix venom. 0.7-ml aliquots were clotted in a humidity chamber for 6-18 h. Cross-linked fibrin was prepared in the same way with the addition of 1 U/ml of preactivated Factor XIII before clotting. Factor XIII purified from human placenta (Behringwerke Hoechst-Roussel, Somerville, NJ) that contained only a chains were activated with 1 U/ml thrombin in 10 mM calcium chloride at 37°C for 30 min, after which the thrombin was inhibited by adding 10 U/ml hirudin (Sigma Chemical Co.). Because fibrinopeptide cleavage by the contortrix enzyme is temperature dependent (29, 31), clots formed with this enzyme were prepared at 4°C, favoring cleavage of FPB, whereas thrombin and reptilase clots were formed at 25°C. All clots were rinsed three times with HBSS containing 1 U/ml hirudin at 25°C before incubation with cells. Supernatants from contortrix clots were prepared by removing the fibrin and centrifuging at 12,000 g for 2 min. The supernatant from a single clot was added to 1 ml of media containing 10 U/ml hirudin and added to the EC. Polypeptide chain composition of the fibrin was assessed after disulfide bond reduction by electrophoresis on 7% SDS polyacrylamide gels (32) after staining with Coomassie blue.

Analysis of fibrinopeptide cleavage. Duplicates of clots used in release assays were formed for quantitative analysis of fibrinopeptide cleavage. For each clot, the fibrin was removed with a wooden stick, and the clot liquor was collected and centrifuged at 12,000 g for 2 min. The supernatants were heated for 30 min at 100°C and then centrifuged for 30 min at 12,000 g to remove denatured, precipitated proteins. Samples of 50 μ l of clot supernatant and 150 μ l of 0.09% aqueous trifluoroacetic acid (TFA) were injected into a C18 analytical reverse phase HPCL column (Radpak; Waters Associates, Milford, MA) connected to a chromatograph (model 8000; Spectra-Physics, Inc., Mountain View, CA) with a compression module (Model RCM-100; Waters Associates). Fibrinopeptides were eluted from the column at a flow rate of 1 ml/min with an isocratic flow phase of 80% aqueous TFA and 20% acetonitrile containing 0.09% TFA. Three peaks could be identified on the basis of comparative migration with standards of FPA, FPB, and phosphorylated FPA prepared from human fibrinogen as described previously (33). Peptide $B\beta 2-14$ (FPB with a deletion at the NH₂ terminus) was identified by amino acid analysis in supernatants of clots prepared with thrombin or contortrix (data not shown). The amount of fibrinopeptide in each sample was determined by quantitative analysis of the peak areas in the HPLC chromatograms of the peptides, which were calibrated against standards of fibrinopeptides. For each clot, the percentage of theoretical yield of FPA represented the sum of both FPA and phosphorylated FPA, whereas the percentage of FPB represented the sum of FPB and $B\beta$ 2-14.

Amino acid analysis of purified peptides. Analysis of the amino acid compositions of the purified peptides and the quantitative determination of standard fibrinopeptides were carried out with an amino acid analysis system (Pico Tag; Waters Associates) at the Cornell University Biotechnology Program Facility. The peptide samples were hydrolyzed and derivatized according to the method of Bidlingmeyer and colleagues (34).

Preparation of fibrinogen B β chain and peptides. To prepare B β chain, fibrinogen was dissolved after reduction and S-carboxymethylation (35) in 0.01 M sodium phosphate, 0.01 M Tris, 8 M urea, and the solution was adjusted to pH 7 with phosphoric acid. The fibrinogen chains were then chromatographed on a column of DEAE-Sephacel (1 × 33 cm) at a flow rate of 16 ml/h using a buffer gradient as described by Stathakis and colleagues (36). Protein containing peaks were pooled, dialyzed against distilled water and lyophilized. The pool containing carboxymethylated B β chains was identified by 7% SDS PAGE and comparison with reduced fibrinogen standard.

A plasmin digest of fibrinogen was prepared using a modification of the methods of Kudryk and colleagues (37). Fibrinogen was diluted to 4 mg/ml in 0.037 M Tris, 0.15 M sodium chloride, 0.1% sodium azide, 0.01 M calcium chloride, pH 7.3, and then incubated for 90 min at 37°C with 100 U/ml streptokinase (Kabi Vitrum, Stockholm). The digest was filtered through a 10,000- M_r cutoff filter (Amicon Corp., Lexington, MA) and the filtrate was found to contain 30 µg/ml protein by Bradford analysis (30). A portion of the filtrate was incubated with 1 U/ml thrombin for 30 min and then treated with 10 U/ml hirudin to inhibit the thrombin activity.

 $B\beta 1-42$ was prepared from plasmic digests of human fibrinogen as described by Skogen et al. (38). HPLC purification was conducted on a 250×10 mm Hi-Pore-318 C18 semipreparative reverse phase column (Bio-Rad Laboratories, Rockville Center, NY) with a biphasic linear 0-25% acetonitrile gradient (38) generated by an automated gradient controller and pump (Model 510; Waters Associates). Absorbance was detected at 214 nm on an absorbance detector (model 441; Waters Associates). Polypeptide-containing peaks were collected, lyophilized, and analyzed for amino acid composition as described above (34). Material from selected peaks was redissolved in a minimum volume of 0.02 M Tris-maleate, 0.1% sodium chloride, 0.01 M calcium chloride, pH 7.4, and treated with 10 U/ml thrombin to identify thrombin-sensitive peptides. After a 1-h incubation, the peptides were rechromatographed on the same column and the peaks produced by the thrombin cleavage, B β 1-14 (FPB), B β 2-14, and β 15-42, were collected, lyophilized, and analyzed for amino acid composition (34).

 β 15-42 was synthetically prepared by a modification of the solidphase peptide synthesis procedure (39, 40) using a peptide synthesizer (Model 430A; Applied Biosystems, Inc., Foster City, CA). The *t*-butoxy-carbonylamino acids were sequentially coupled as symmetric anhydrides onto a phenyl-acetamidomethyl (OCH₂PAM) resin (Applied Biosystems, Inc.) with double coupling cycles used for all arginine residues. The remainder of the synthesis, purification, and characterization of the peptide were conducted as described (40). Peptide β 15-18 (GHRP) was purchased from Sigma Chemical Co.

Cell culture and immunofluorescence. Human umbilical vein EC were grown in culture as described elsewhere (41, 42). Primary cultures plated on glass coverslips were grown in McCoy's 5A media (Flow Laboratories, Inc., McLean, VA) containing 20% fetal bovine serum (Hyclone Laboratories, Logan, UT). Fibrin stimulation of vWf release with preformed clots was carried out as described (8) except that all incubations were conducted at 25°C instead of 37°C because the contortrix-formed fibrin dissociated above 25°C (31). For vWf release stimulation assays using peptides, all solutions were made in McCoy's 5A medium with 20% FCS and 10 U/ml hirudin. Coverslips were placed in individual 16-mm culture wells in a 24-multiwell culture plate (Corning Glass Works, Corning, NY). Each coverslip was covered with 200 μ l of peptide solution and incubated at 37°C for varying times. Indirect immunofluorescence staining of cells with anti-vWf antiserum and quantitation of vWf release followed procedures described previously (42).

For metabolic labeling, EC were grown in plastic flasks to 80% confluency in McCoy's 5A media and then 25 μ Ci/ml of [³⁵S]cysteine (Amersham Corp., Arlington Heights, IL) was added and the cells cultured an additional 3 d. After a 6-h chase with unlabeled media, the cultures were washed three times with Hank's buffer and then incu-

bated with potential secretagogues in media containing 10 U/ml hirudin for 3 h. The media was then collected and vWf was immunopurified as described (43) using rabbit anti-human vWf antiserum (Calbiochem-Behring Corp., La Jolla, CA). The immunopurified vWf was electrophoresed after disulfide reduction on SDS 5% polyacrylamide gels (32) followed by autoradiography, and release was quantitated by densitometric scanning of the 220-kD chain (Quick-Scan, Jr., TLC; Helena Laboratories, Beaumont, TX).

Statistical analysis. Comparison of means was preformed using the two-tailed t test. Variance is described as \pm SEM.

Results

EC stimulation by fibrin. Cross-linked and non-cross-linked fibrin were prepared using thrombin, reptilase, or contortrix to remove fibrinopeptides selectively, and the polypeptide chain composition was characterized by SDS-PAGE (Fig. 1). The γ chains for all three types of non-cross-linked fibrin demonstrated the same mobility as did fibrinogen, consistent with absence of cleavage in this chain. Upon cross-linking (Fig. 1), the γ chains dimerized, as evidenced by the disappearance of monomers and appearance of a $\gamma\gamma$ band not found in noncross-linked samples. Cleavage of FPB from the $B\beta$ chain was seen with thrombin and contortrix enzyme as an increased migration of the β chain as compared with the B β chain present in fibrinogen and reptilase fibrin. Cleavage of FPA from the thrombin and reptilase fibrin also appeared as an increased mobility of the α chains compared with the A α chain fibrinogen standard. Cross-linking of the α chain led to a decrease in



Figure 1. SDS-PAGE of fibrin. Fibrin was prepared using thrombin, reptilase, or contortrix procoagulant in the presence or absence of Factor XIII_a for cross-linking. After dissolution with reduction, the polypeptide chains were separated by 7% SDS-PAGE. In the noncross-linked fibrin, γ chains are present as monomers that are replaced by γ dimers in cross-linked samples. B β chains in fibrinogen and reptilase fibrin demonstrate slower migration compared with the β chains present in fibrin made with contortrix procoagulant or thrombin. Similarly, $A\alpha$ chains in the fibrinogen are replaced by faster migrating α chains in fibrin prepared with thrombin or reptilase. In the cross-linked fibrin, heterogenous high relative molecular mass cross-linked polymers are formed from the $A\alpha$ chains, leading to the decreased intensity of the $A\alpha$ band, and the appearance of bands migrating more slowly than the γ - γ bands. protein present in the monomeric bands and the formation of higher molecular weight α -polymer bands migrating more slowly than the $\gamma\gamma$ chain, with some failing to enter the gel. FPA cleavage in the contortrix clot could not be readily demonstrated because this enzyme cleaved the proteolytically sensitive, carboxy-terminal end of the α chain, also resulting in increased mobility (29). The extent of fibrinopeptide cleavage was determined by quantitative HPLC analysis (Table I). Thrombin cleaved both FPA and FPB completely, whereas reptilase cleaved only FPA but not FPB. The contortrix enzyme preferentially cleaved FPB at 4°C producing fibrin, from which 90% of FPB but only 21% of FPA was removed.

The stimulatory abilities of the fibrins was assessed after immunofluorescent staining of EC for vWf after a 10-min incubation with fibrin (Table I, Fig. 2). Control cells demonstrated diffuse perinuclear staining of the vWf reflecting the presence of the protein in the endoplasmic reticulum and Golgi apparatus, and also showed staining of the rod-shaped cytoplasmic storage granules, the Weibel-Palade bodies (Fig. 2. arrowheads). Control cells in both the presence and absence of factor XIII_a demonstrated a basal level of vWf secretion (Table I). After stimulation with non-cross-linked or cross-linked reptilase-made clots the vWf distribution was unchanged from untreated controls (Table I, Fig. 2). In contrast, after stimulation with non-cross-linked or cross-linked thrombin- or contortrix-made fibrin, the staining pattern of the cells changed, and the Weibel-Palade bodies were replaced by bright patches of cell surface vWf staining (Fig. 2, arrows) with nearly complete release (Table I). Neither contortrix venom alone nor the supernatant from the contortrix clots stimulated release above background. When clots were made using 5×10^3 U/ml thrombin at 4°C for 6 h, HPLC analysis revealed that 53% of FPA and only 10% of FPB was removed. These clots were unable to stimulate the EC, with 10 and 26% of the cells secreting after treatment with a non-cross-linked or cross-linked clot, respectively. These data demonstrate a correlation of FPB cleavage with fibrin stimulation ability, but no apparent effect of either FPA cleavage or fibrin cross-linking.

 Table I. Analysis of Fibrinopeptide Cleavage and vWf Secretion
 Stimulation by Preformed Fibrin Clots

	Percen re Facto Facto ryme cleaved* cleaved* Cross-linke		Percent cells that have released vWf [‡]		
			Factor XIIIa (1 U/ml)		
Fibrinogen cleaving enzyme		Cross-linked	Non-cross-linked		
None	_	_	4±1	7±0.2	
Reptilase (1.1 U/ml) Thrombin	99±2	0±0	11±1	8±1	
$(5 \times 10^{-3} U/ml)$	105±3	94±5	79±2	92±1	
Contortrix	21±1	90±5	83±8	80±2	

* Fibrinopeptide cleavage was determined by HPLC analysis of the clot liquors. Results represent the mean percentage of four to six determinations±SE.

[‡] Primary EC cultures were incubated for 10 min at 25°C with preformed clots in media with 10 U/ml hirudin and the percent of released cells was determined by immunofluorescent staining. Results represent the mean±SE of 5-28 determinations.



Figure 2. Immunofluorescent localization of vWf in EC before and after treatment with fibrin. EC grown on glass coverslips were exposed to fibrin made using thrombin, reptilase, or contortrix procoagulant. After a 10min stimulation, the cells were fixed, permeabilized, and stained with anti-vWf antiserum. Control cells and cells exposed to fibrin made with reptilase (top and bottom left, respectively) demonstrated the perinuclear staining of vWf in the endoplasmic reticulum and cytoplasmic rod-shaped organelles, the Weibel-Palade bodies (arrowheads). In the cells treated with thrombin and contortrix-fibrins (top and bottom right, respectively), the Weibel-Palade bodies are replaced by bright patches of released vWf on the cell surface. Bar, 10 μ m.

Endothelial cell stimulation by $B\beta$ chain and $B\beta$ chain peptides. To define further the fibrin structure needed to stimulate release, we prepared $B\beta$ chain and selected peptides and examined their effect on EC. Intact $B\beta$ chain caused significant stimulation (P < 0.05) above background only at 200 nM, with $26\pm6\%$ of the cells showing patches of release (Fig. 3). After treatment with thrombin, however, β chain caused release above background (P < 0.01) at 20 and 200 nM concentrations, stimulating 36 ± 9 and $60\pm7\%$ SE, respectively. The release after thrombin cleavage was significantly above that seen with the uncleaved $B\beta$ chain at 20 nM (P < 0.01) and 200 nM (P < 0.025), demonstrating a dependence upon FPB cleavage for stimulation of secretion.

FIBRIN (Reptilase)

An $M_r < 10,000$ fraction of a plasmic digest of fibrinogen that contained B β 1-42 did not stimulate vWf release after a 3-h incubation with EC (Table II). However, the same preparation stimulated 73% release after incubation with thrombin, indicating that thrombin-cleaved plasmic peptides retained activity. B β 1-42 was purified from the $M_r < 10,000$ fraction by HPLC, eluting as a single peak after rechromatography (Fig. 4 *A*), and its identity was confirmed by amino acid analysis (Table III). After cleavage by thrombin (10 U/ml), two major peaks were produced (Fig. 4 *B*) and amino acid analysis of these peaks confirmed their identity as FPB and β 15-42 (Table III). Variations from theoretical yields of amino acid residues of B β 1-42 could be explained by the presence of small contaminating peptides seen as residual peaks eluting on HPLC in the B β 1-42 area after thrombin cleavage (Fig. 4 B). Cells incubated with 0.1 mM concentrations of B β 1-42 did not stimulate release significantly above control (Table IV), but release was stimulated at 3 h by either purified or synthetic peptide at concentrations of 0.1 and 1 mM (P < 0.005). No release was seen with FPB or with the peptide GHRP representing the four NH₂-terminal amino acid residue of the β chain.

To confirm release with β chain and with β 15-42, EC were metabolically labeled, and released vWf was immunopurified and characterized by SDS-PAGE (Fig. 5). The vWf released by A23187, β chain, or β 15-42 was composed of equal amounts of the mature 220-kD subunit and 100-kD propolypeptide, confirming its release from Weibel-Palade bodies. Compared with the complete release by A23187, 200 nM β chain and 100 μ M β 15-42 stimulated 36 and 23%, respectively.

Discussion

FIBRIN (Contortrix)

The results demonstrate that the stimulation of vWf release from EC by fibrin requires cleavage of FPB from fibrinogen. This was established by comparing the effects of fibrin pre-



Figure 3. B β chain stimulation of vWf release from endothelial cells, before and after cleavage with thrombin. EC were incubated for 3 h with 10 U/ml hirudin containing solutions of $B\beta$ chain before (open bars) or $B\beta$ chain after thrombin cleavage (hatched bars) at concentrations of 2, 20, and 200 nM. Cells were fixed, permeabilized, and stained for vWf, and the percent of cells demonstrating patches of released vWf was determined. Cells incubated in culture medium with hirudin demonstrated a basal secretion of

11±2%. Stimulation with B β chain was significantly above control (P < 0.05) only at 200 nM. Stimulation with thrombin treated B β chain was above control for 20 nM (P < 0.025) and 200 nM concentrations (P < 0.01). The differences in stimulation at 20 nM and 200 nM for the B β chains before and after thrombin cleavage were both statistically significant (P < 0.05 and < 0.025, respectively). Bars represent the mean of three to five determinations±SE.

pared with three enzymes differing in specificity for fibrinopeptide cleavage. Release of vWf was stimulated by fibrin prepared with either thrombin, which cleaves both FPA and FPB, or with the contortrix procoagulant, which cleaves primarily FPB (Table I). Release did not occur with exposure of EC to fibrin prepared with reptilase, which removes only FPA (Table I), confirming our prior report (8). The results further indicate that stimulation of release is independent of Factor XIII_a-mediated fibrin cross-linking, as both non-cross-linked and cross-linked fibrin were stimulatory after FPB cleavage (Table I). The stimulation of release by fibrin prepared with contortrix procoagulant confirms previous findings indicating that fibrin induced release was not caused by small amounts of fibrin-bound thrombin (8).

Table II. vWf Release Stimulated by Plasmic Peptides of Fibrinogen

Stimulant	% Released cells*
Control (media alone)	10±5
Thrombin (1 U/ml)	99±1
Thrombin $(1 \ U/ml)$ + hirudin $(10 \ U/ml)$	7±2
Plasmic peptides of fibrinogen $(30 \ \mu g/ml)^{\dagger}$	20±4
Plasmic peptides of fibrinogen ($30 \ \mu g/ml$), thrombin ($1 \ U/ml$), and hirudin ($10 \ U/ml$) ^{‡§}	73±13

* Primary EC cultures were incubated for 3 h in culture medium to which stimulatory reagents were added. The percentage of released cells was determined after immunofluorescence staining. Results represent the mean of three to five observations±SE.

[‡] TCA soluble; pass M_r 10,000 filter.

[§] Peptides were incubated at 37°C with thrombin for 30 min, and then the thrombin was incubated for 10 min with hirudin.



Figure 4. HPLC elution patterns of peptides derived from plasma digestion of fibrinogen. Fibrinogen was digested for 30 min at 37°C with 0.02 U/ml plasmin, the large molecular weight proteins precipitated with 0.05% TCA and the supernatant filtered through a 10,000 Mr cut-off membrane after centrifugation. The peptides were separated by HPLC on a RP-C18 318 semipreparative column (Bio-Rad Laboratories) as described by Skogen et al. (38) and peaks containing $B\beta$ 1-42 were identified. The pool of $B\beta 1-42$ was rechromatographed (A) and was composed of a single peak. Treatment of this sample with 10 U/ml thrombin for 2 h at 25°C (B) produced two major peaks, found by amino acid analysis to be β 15-42 and FPB.

The results with purified $B\beta$ chain and $B\beta$ chain peptides implicate a site near the NH₂ terminus of the β chain in mediating vWf release. The $B\beta$ chain showed minimal activity at 200 nM, whereas significant release occurred at 20 nM after thrombin cleavage (Fig. 3). The finding that a low relative molecular mass fraction of a plasmic digest of fibrinogen stimulated release after thrombin cleavage (Table II), is also consistent with involvement of a site at the β chain NH₂ terminus, because $B\beta$ 1-42 is cleaved early during plasmic degradation of fibrinogen (38) and contains the arg14-gly15 bond that is sen-

Table III. Amino Acid	Analysis of Fibrinogen!
Degradation Products	

	B β1	-42*	β15	β15-42 [‡] FF	₽B ^{\$}	
Amino acids	Observed	Expected	Observed	Expected	Observed	Expected
ASX	4.7	4	1.1	1	2.8	3
GLX	5.1	5	2.2	2	3.0	3
SER	2.6	- 3	2.3	2	1.0	1
GLY	5.2	6	4.7	4	2.0	2
HIS	1.0	1	1.0	1	0	0
ARG	4.3	5	4.0	4	1.1	1
THR	1.1	0	0.3	0	0	0
ALA	3.3	3	1.8	2	1.0	1
PRO	5.2	6	5.4	6	0	0
TYR	0.6	1	0.6	1	0	0
VAL	1.3	1	0.2	0	1.0	1
MET	1.9	0	0.1	0	0	0
CYS	0.1	0	0.2	0	0	0
ILE	0.6	1	0.9	1	0	0
LEU	2.7	2	1.8	2	0	0
PHE	2.1	2	0.1	0	1.9	2
LYS	1.6	2	1.7	2	0	0

* Average of 11.

[‡] Average of 5. [§] Average of 5.

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Table IV. Release of vWf from EC Stimulated by Peptides Derived from $B\beta$ Chains

Stimulant	% Released cells*
Control (medium alone)	11±2
FPB (Bβ1-14)	15±3
GHRP (β15-18)	7±1
Ββ1-42	17±4
β15-42 (0.1 mM)	26±2
$\beta 15-42 (1 mM)$	47±3

* EC were incubated for 3 h with peptides in medium containing 10 U/ml hirudin. The peptide concentration was 0.1 mM except as noted. The percentage of cells with release patches was determined after immunofluorescence staining. Means \pm SE of four to six determinations. Stimulation by β 15-42 was significantly above control values (P < 0.005) at concentrations of 0.1 and 1 mM of purified or synthetic peptide.

sitive to cleavage by thrombin. To confirm the involvement of a site at the β chain NH₂ terminus, we purified B β 1-42, β 15-42, and FPB (Fig. 4) and found that only β 15-42 had stimulatory activity (Table IV). Release was confirmed by immunopurification of released vWf after exposure of EC to β chain or β 15-42 (Fig. 5). The released vWf was composed of mature, highly processed subunits, and released in equal amount to the propolypeptide (Fig. 5), indicating that it derived from Weibel-Palade bodies (20).

None of the soluble derivatives was as active in stimulating release as intact fibrin, as these preparations stimulated less at 3 h than intact fibrin stimulated at 10 min. One potential explanation for this finding is that the complete active site includes structures contained on the α or γ chain present in intact fibrin but absent in the β chain peptides. A second explanation for the slower release with peptides is that the necessary conformation of the β chain for stimulation is stabilized in intact fibrin, and this is consistent with the greater activity of the whole β chain, which was active at a concentration as low



Figure 5. SDS-PAGE of metabolically labeled vWf released from endothelial cells. EC were metabolically labeled with [35S]cysteine, chased for 6 h in nonradioactive media, and then washed. The cultures were then incubated for three hours with medium containing 10 U/ml hirudin (lane 1), or medium with 10 U/ml hirudin containing 10 µM A23187 (lane 2), 200 nM β chain (lane 3), or 100 μ M β 15-42 (lane 4). After electrophoresis and autoradiography, two bands were identified representing mature vWf (220 kD) and the vWf propolypeptide (100 kD).

as 20 nM (Fig. 3). Stimulation of release by the B β chain at the highest concentration (Fig. 3) may indicate that it can occasionally assume the necessary conformation for stimulation. Finally, it may be that maximum release requires cross-linking of multiple EC surface receptors that can occur with polymerized fibrin but not with soluble polypeptide chains or peptides.

Several peptides resulting from thrombin or plasmin cleavage of the $B\beta$ chain NH_2 terminus have been shown to be functionally active. Thrombin cleavage of the arg14-gly15 bond creates a new β chain NH₂ terminus, and the first four amino acid residues, gly-his-arg-pro, influence fibrin polymerization (44, 45). Plasmin rapidly cleaves $B\beta 1-42$ from fibrinogen or β 15-42 from fibrin and can also liberate the pentapeptide β 43-47 (46). FPB and plasmic peptides of fibrinogen and fibrin have been shown to cause EC shape change and stimulation of migration in culture (47). Additional effects on EC include the reported vasoconstrictor activity of FPB (46) and the vasodilation and increased vascular permeability stimulated by β 43-47 (46, 48). FPB and B β 1-42 have effects on other cell types including the stimulation of migration of neutrophils (49-51), monocytes (52), and fibroblasts (49, 50). Plasmic peptides of fibrinogen, including β 43-47 inhibit thymidine uptake in stimulated lymphocytes (53, 54) suggesting that they have immunosuppressive effects. The findings in this report demonstrate that removal of FPB is necessary for expression of vWf releasing activity since neither intact fibrinogen nor $B\beta 1-42$ had activity. This indicates that EC specifically recognize the amino terminus of the β chain, possibly by a receptor-mediated mechanism. The recent findings by Chen et al. (55) that β 15-42 inhibits platelet aggregation and binding to activated platelets is consistent with this hypothesis. Together, the evidence suggests an important role for sites near the NH₂ terminus of the B β or β chain in modulating the cellular changes accompanying hemostasis and inflammation at sites of fibrin deposition.

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