Trigramin: Primary Structure and Its Inhibition of von Willebrand Factor Binding to Glycoprotein IIb/IIIa Complex on Human Platelets†

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ABSTRACT: Trigramin, a naturally occurring peptide purified from *Trimeresurus gramineus* (*T. stejnegeri formosensis*) snake venom, inhibits platelet aggregation and the binding of ^{125}I-fibrinogen to ADP-stimulated platelets ($K_i = 2 \times 10^{-8}$ M) without affecting the platelet-release reaction. ^{125}I-trigramin binds to ADP-stimulated and to chymotrypsin-treated normal platelets but not to thrombasthenic platelets. ^{125}I-trigramin binding to platelets is blocked by monoclonal antibodies directed against the glycoprotein IIb/IIIa complex and by Arg-Gly-Asp-Ser (RGDS) [Huang et al. (1987) *J. Biol. Chem. 262*, 161]. We determined the primary structure of trigramin, which is composed of a single polypeptide chain of 72 amino acid residues and six disulfide bridges. The molecular weight of trigramin calculated on the basis of amino acid sequence was 7500, and the average p*I* was 5.61. An RGD sequence appeared in the carboxy-terminal domain of trigramin. An amino-terminal fragment (7–33) of trigramin showed 39% homology with a region (1555–1581) of von Willebrand factor (vWF). Trigramin also showed 36% identity in a 42 amino acid overlap and 53% identity in a 15 amino acid overlap when compared with two adhesive proteins, collagen α_1 (I) and laminin B_1, respectively. Trigramin blocked binding of human vWF to the glycoprotein IIb/IIIa complex in thrombin-activated platelets in a dose-dependent manner. Reduction of trigramin resulted in a marked decrease in its ability to block vWF binding to human platelets. In conclusion, our data suggest that the biological activity of trigramin may depend upon the presence of an RGD sequence, the secondary structure of the molecule, and perhaps some other sequences that it shares with adhesive proteins.

The interaction of fibrinogen with specific platelet receptors associated with glycoprotein IIb/IIIa (GPIIb/IIIa) is essential for platelet aggregation [for review see Peerschke (1985) and Phillips et al. (1988)]. When platelets are stimulated by agonists such as ADP (Marguerie et al., 1979; Bennett & Vilaire, 1979; Kornecki et al., 1981) or thrombin (Hawiger et al., 1980; Plow & Marguerie, 1980), fibrinogen receptors associated with the GPIIb/IIIa complex become exposed on the platelet surface, resulting in fibrinogen binding and subsequent platelet aggregation. However, the mechanism of fibrinogen receptor exposure on the platelet membrane and the molecular events associated with the interaction of fibrinogen with GPIIb/IIIa are not yet elucidated. There is evidence that fibrinogen reacts with an epitope involving both components of the complex (Coller et al., 1983; McEver et al., 1983) and exposure of this epitope depends on the conformation of the GPIIb/IIIa complex (Shattil et al., 1985). Calcium is essential for the formation of the GPIIb/IIIa epitope interacting with fibrinogen (Jennings & Phillips, 1982; Brass et al., 1985). Monoclonal antibodies directed against the GPIIb/IIIa complex (Coller et al., 1983; Pidard et al., 1983; Bennett et al., 1983) and synthetic peptides representing putative platelet binding sites on the fibrinogen molecule (Kloczewiak et al., 1984; Gartner & Bennett, 1985; Plow et al., 1985) specifically inhibit binding of fibrinogen to the GPIIb/IIIa complex. In addition to fibrinogen, the GPIIb/IIIa complex can serve as a receptor for three additional plasma proteins: fibronectin (Plow & Ginsberg, 1981), von Willebrand factor (Fujimoto et al., 1982; Ruggeri et al., 1983; Timmons et al., 1984; Gralnick et al., 1984), and vitronectin (Pytela et al., 1986).

Trigramin, a naturally occurring peptide purified from *Trimeresurus gramineus* snake venom, blocked platelet aggregation and binding of ^{125}I–fibrinogen to ADP-stimulated platelets ($K_i = 2 \times 10^{-8}$ M) without affecting the platelet-release reaction (Huang et al., 1987). ^{125}I-trigramin bound to ADP-stimulated and to α-chymotrypsin-treated platelets. Binding of trigramin to platelets was blocked by monoclonal antibodies directed against the GPIIb/IIIa complex and by high concentrations of RGDS and of the fibrinogen γ-chain COOH-terminal pentadecapeptide (Huang et al., 1987). We postulated that the biological activity of trigramin depends on the presence of RGD in its molecule and on its secondary structure since its biological activity was lost after reduction.

In this paper we describe the complete primary structure of trigramin, its homology to three other proteins, and its inhibitory effect on von Willebrand factor binding to platelets.

Materials and Methods

Trigramin was prepared from *T. gramineus* (*T. stejnegeri formosensis*) snake venom according to the method described previously (Ouyang & Huang, 1983; Huang et al., 1987). In brief, the preparatory procedure consisted of DEAE-Sephadex A-50 column chromatography and gel filtration on Sephadex G-75 and G-50 columns. Finally, the crude trigramin was

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purified by reverse-phase HPLC to remove a phospholipase A contaminant. The purified trigramin showed a single band on SDS–PAGE[1] and contained a single NH_2-terminal sequence, Glu-Ala-Glu. Trigramin appears to constitute 0.5% of the total proteins in snake venom.

Amino acid analysis was carried out by the Protein Chemistry Facility of the Wistar Institute, University of Pennsylvania, under the direction of Dr. D. W. Speicher. Hydrolysis (vapor phase, 6 N HCl with 1% phenol, 1 h at 160 °C) was followed by a manual derivatization with phenyl isothiocyanate and separation of amino acids by reverse-phase HPLC.

Pyridylethylation of trigramin was carried out by adding 1 μL of vinylpyridine to the reduced protein (50 μg in 99 μL of 6 M guanidine hydrochloride, 4 mM EDTA, 0.1 M Tris-HCl, pH 8.5, and 4 mM dithiothreitol). The reaction mixture was incubated 2 h at 22 °C in the dark under argon first for reduction and then a further 2 h for pyridylethylation. Modified protein was isolated free of reagents by reverse-phase HPLC in 0.1% trifluoroacetic acid with acetonitrile as organic modifier.

Automated NH_2-terminal sequencing was performed on a gas-phase sequencer (Applied Biosystems Inc., Model 470A) coupled to an on-line PTH analyzer (Applied Biosystems Inc., Model 120A). These instruments are operated routinely by the Macromolecular Analysis and Synthesis Laboratory of the Temple University Health Sciences Center. Standard protocols of the manufacturer were followed with regard to both Edman degradation and separation of PTH-amino acids by HPLC. Cysteine was detected as *S*-(pyridylethyl)cysteine.

Strategy of Sequencing. Automated NH_2-terminal sequencing of *S*-(pyridylethyl)- (*S*-PE-) trigramin yielded 35 residues from the NH_2 terminus. Cleavage of *S*-PE-trigramin was undertaken to generate peptide fragments. Enzyme digests (see below) were terminated by acidification to pH <3 and peptides isolated by reverse-phase HPLC on a wide-pore Vydac C-18 column (25 × 0.4 cm) (The Separations Group, Hesperia, CA) equilibrated in 0.1% trifluoroacetic acid. The HPLC system consisted of two Waters Model 510 pumps, a Waters Model 680 gradient controller, an LKB Uvicord S detector with 206-nm filter, and a gradient (1%/min) of acetonitrile. Manualy collected peaks were either dried in a Speedvac (Savant Instruments) or analyzed directly on the sequencer. Concentrations were estimated from the areas of peaks detected at 206 nm. Typically, 200–500 pmol of peptide was used to define up to 20 residues.

Cleavage with chymotrypsin (Worthington Biochemical Corp.) was carried out at pH 8 for 1.5 h at 37 °C with an enzyme to protein ratio of 1:100 by weight. Chymotrypsin cleaved *S*-PE-trigramin primarily at *S*-PE-cysteine residues under these conditions.

Cleavage with trypsin (TPCK treated, Worthington Biochemical Corp.) was carried out at pH 8 for 17 h at 37 °C with an enzyme to protein ratio of 1:50 by weight.

Cleavage with Staphylococcus aureus (V8 strain) protease (Pierce Chemical Co.) was carried out in 15 mM sodium phosphate, pH 7.8, and 5 mM EDTA. An enzyme to substrate ratio of 1:16 by weight was used, and cleavage was allowed to proceed for 21 h at 37 °C.

Human von Willebrand factor (vWF) was purified by a modification of the method of Newman et al. (1971) and Switzer et al. (1976). The preparation involved cryoprecipitation and PEG-6000 precipitation of normal plasma. The precipitate was then chromatographed on a Sepharose CL-4B column. The void volume fraction with the highest OD at 280 nm was collected, concentrated by using PEG-6000, and dialyzed against 0.01 M phosphate and 0.15 M NaCl, pH 6.5. The protein content was determined by the method of Lowry et al. (1951). The preparation of vWF showed a single band with an apparent molecular weight of 220K on a 5% SDS–polyacrylamide gel under reducing conditions.

Labeling of Human von Willebrand Factor. Human vWF was labeled with ^{125}I by the iodogen method (Markwell & Fox, 1978). vWF (0.8–1 mg) was dialyzed against 0.1 M phosphate buffer, pH 7.0. NaI was added to the sample to a final concentration of 8 μM, followed by 400 μCi of $Na^{125}I$ (New England Nuclear, Boston, MA; specific activity = 17 Ci/mg). The solution was transferred to a 5-mL capped septum vial that had been coated with 50 μg of iodogen. The vial was swirled gently for 10 min at room temperature. Labeling was stopped by transferring the sample to a vial containing 0.1 ml of 0.1 M NaI solution in water. The sample was passed over a column (1 × 10 cm) of Sephadex G-25 equilibrated in phosphate-buffered saline. Void volume fractions with the highest specific activity were pooled. Incorporation of ^{125}I was 50–60%, and >95% of the radioactivity in the labeled protein was precipitable with trichloroacetic acid. The labeled human vWF contained about 1.3×10^5 cpm/μg.

Preparation of Human Platelet Suspensions. Blood was obtained from normal healthy individuals who denied taking any medications within the previous 2 weeks. The donors donated blood after signing an informed consent in agreement with the Helsinki guidelines. Blood collected in acid citrate dextrose (1/10 v/v) was centrifuged at 150 g at room temperature for 10 min to obtain platelet-rich plasma. Washed platelet suspensions were prepared according to the method of Mustard et al. (1972). Platelets were finally suspended in Tyrode's albumin solution (pH 7.35) containing 3.5 mg/mL bovine serum albumin (Sigma fraction V).

Binding of ^{125}I-vWF to Platelets. Ligand–platelet binding studies were performed by the previously described method (Niewiarowski et al., 1981) with slight modification. In brief, the incubation mixture was composed of 420 μL of platelet suspension (about 5×10^8 platelets/mL), 10 μL of ^{125}I-vWF (5 μg/mL), 10 μL of α-thrombin (0.5 unit/mL) or 10 μL of ristocetin (0.75 mg/mL, Sigma), and 10 μL of Tyrode's solution or 10 μL of inhibitor solution (trigramin, synthetic peptides, monoclonal antibodies, EDTA). The concentrations in parentheses are the final ones. In a typical experiment, ^{125}I-vWF was added simultaneously with inhibitors to platelet suspensions at 22 °C, 3 min before addition of agonist. Following the addition of ristocetin the platelet suspension was gently shaken and incubated for another 10 min. Following addition of thrombin the platelet suspension was incubated for 5 min and thrombin was neutralized with a 5-fold molar excess of hirudin (Sigma Chemical Co.). Following the addition of hirudin a further 15-min incubation was performed before centrifugation. Then 400 μL of the platelet suspension was centrifuged through silicone oil at 15000*g* in an Eppendorf centrifuge. The tips of the centrifuge tubes were cut off and counted for bound ^{125}I-vWF. The nonspecific binding of ^{125}I-vWF was measured in the absence of thrombin or ristocetin. The amount of ^{125}I-vWF bound in the absence of inhibitor was considered as 100%. The inhibitory effect of trigramin or peptides on the binding of ^{125}I-vWF to platelets was expressed as IC_{50} (molar concentration of compound causing 50% inhibition), calculated by means of regression analysis.

[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; *S*-PE, *S*-(pyridylethyl); CHT, chymotrypsin; TRY, trypsin; vWF, von Willebrand factor; PEG, poly(ethylene glycol).

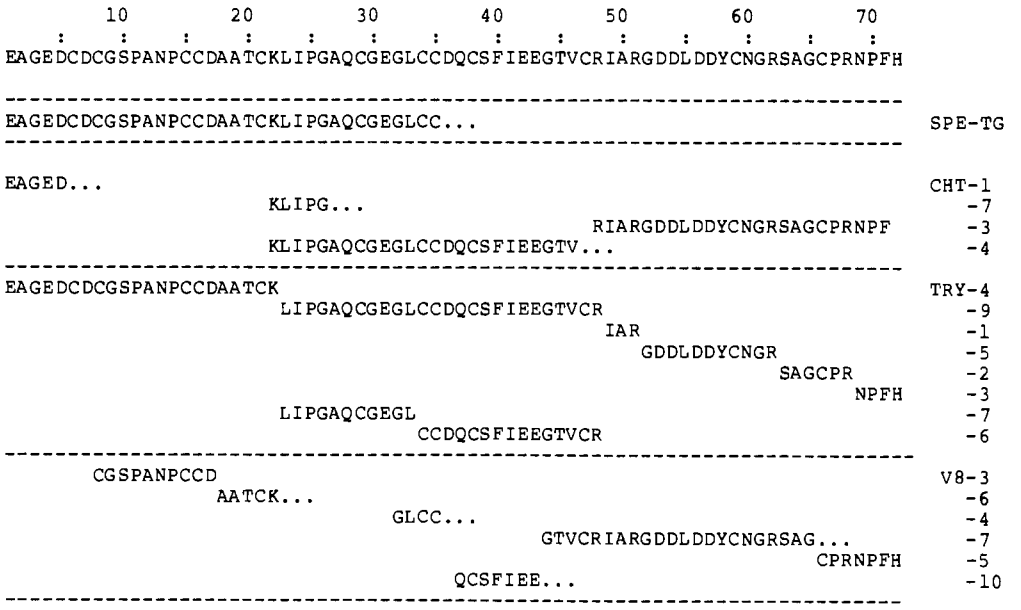

FIGURE 1: Amino acid sequence of trigramin obtained from the venom of *T. gramineus*. The first 35 NH_2-terminal amino acid residues were determined by Edman degradation and then confirmed by analysis of the fragments produced by the proteolytic enzymes chymotrypsin (CHT), trypsin (TRY), and *S. aureus* V8 protease (V8). Each protease produced several fragments under different conditions described in the text. *S*-PE-TG denotes *S*-(pyridylethyl)trigramin. Dots correspond to the undetermined residues. The numbers 1–10 represent fragments produced. All peptide sequences (some not shown) were consistent with the structure presented.

Monoclonal Antibodies. The monoclonal antibodies AP_2 (recognizing the GPIIb/IIIa complex; Pidard et al., 1983) and AP_1 (recognizing GPIb; Montgomery et al., 1983) were gifts of Drs. P. Newman and T. Kunicki (Milwaukee Blood Center, Milwaukee, WI). Monoclonal antibody A_2A_6 (recognizing GPIIb/IIIa complex; Bennett et al., 1983) was donated by Dr. J. S. Bennett (University of Pennsylvania Hospital, Philadelphia, PA).

Synthetic Peptides. RGDS was supplied by Peninsula Laboratories, Inc., Belmont, CA; the COOH-terminal tyrosylpentadecapeptide of fibrinogen γ-chain YGQQHHLGGAKQAGDV was custom synthesized by Peninsula Laboratories, Inc., Belmont, CA. All peptides were dissolved in 0.15 M NaCl.

RESULTS

Sequencing of Trigramin. Amino acid analysis revealed in trigramin a high cysteine content but no methionine. HPLC-purified trigramin was pyridylethylated to convert cysteine to *S*-(pyridylethyl)cysteine (*S*-PE-Cys), a derivative stable during Edman degradation and one easily resolved from other PTH-amino acids in a standard HPLC separation. Intact *S*-PE-trigramin was subjected to NH_2-terminal sequencing, which yielded 35 unambiguous residues (Figure 1). At each cycle the predominant residue was accompanied by up to 20% of the next residue in the sequence. About 20% of the material therefore appeared to lack the first residue, presumably as a result of proteolytic cleavage. The protease responsible for this cleavage was not identified.

Further sequencing depended on deliberate proteolytic cleavage of *S*-PE-trigramin and isolation of fragments by reverse-phase HPLC. The first enzyme used was chymotrypsin (CHT). Manually collected peaks were numbered in the order of their elution from the column and subjected to NH_2-terminal sequencing, with the results shown in Figure 1. CHT-1 was identified as the NH_2 terminus and therefore was not further characterized. CHT-3 and -4 were major peptides isolated from the digest. Most CHT cleavages were observed at *S*-PE-Cys, presumably because of the pyridine ring that had been introduced.

Cleavage of *S*-PE-trigramin with trypsin (TRY) yielded eight fragments which accounted for >95% of the material in the chromatogram of the digest. All but one of the fragments resulted from cleavage at Arg or Lys; the exception was cleavage at Leu-33. Cleavage with *S. aureus* V8 protease was also undertaken in the hope of finding an overlap peptide. Sequences of the peptides isolated are shown in the lower part of the Figure 1. In addition to confirming the existing composite sequence, a peptide (V8-7) was found that established the link between the two larger sequences obtained by chymotryptic cleavage (CHT-3 and CHT-4). One anomalous cleavage (at Gly-65) was noted. No paired acidic residues were targets for cleavage.

The first evidence for the carboxy-terminal sequence was drawn from the *composite sequence* constructed from all the fragments that were detected in proteolytic digests (Figure 1). None of the enzymes used is expected to cleave at histidine, suggesting that the COOH-terminal histidine detected in the tryptic and V8 protease peptides is indeed the COOH-terminal residue of the whole molecule.

Since short peptides sometimes are not sequenced completely by NH_2-terminal methods, the amino acid composition of the tryptic peptide TRY-3 was determined. The molar composition (mean of two analyses) was as follows: Asx, 0.97; His, 0.94; Pro, 1.00; and Phe, 0.97, with no other residue greater than 0.07. This result showed that the peptide had in fact been completely sequenced and that no residues had been lost on the sequencer.

Finally, direct evidence for the COOH-terminal sequence was sought by cleavage with carboxypeptidase Y. *S*-(Pyridylethyl)trigramin was incubated with 5% w/w of enzyme in 0.1 M pyridine and 0.3 M acetic acid, pH 5.5, at 37 °C. After varying times, aliquots were withdrawn into pyrolyzed glass tubes, stored 45 min on ice, heated 30 s at 100 °C, and dried. Amino acids released were quantitated by amino acid analysis, using the recovery of 250 pmol of hydroxyproline to correct for losses. The results obtained in one of two experiments performed are shown in Table I.

Sulfhydryl/Disulfide Content of Trigramin. Two aliquots of a trigramin solution was dried and S-pyridylethylated, one

Table I: Release of Amino Acids (pmol per Sample) from *S*-PE-trigramin by Carboxypeptidase Y

amino acid detected	time of incubation		
	10 min	30 min	60 min
H	78	141	209
F	47	159	244
P	9	70	194
N	0	19	65

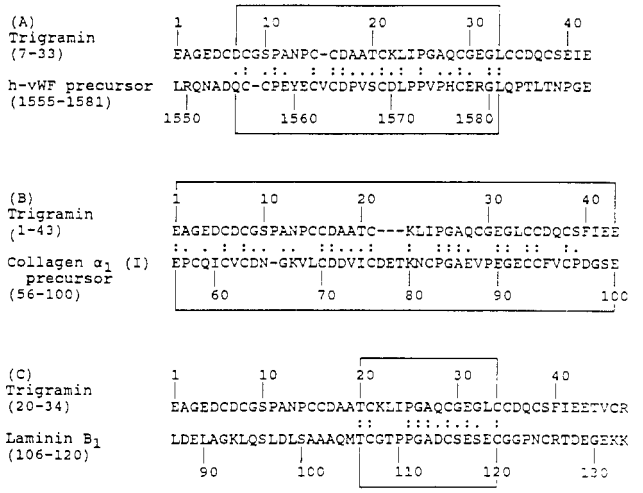

FIGURE 2: Amino acid homology between trigramin and human von Willebrand factor (vWF) precursor (A), collagen α_1 (I) chain precursor (B), and mouse laminin B_1 (C). Identical amino acids are indicated by double dots, and conservative substitution is indicated by single dots. Amino acid sequences use the standard single-letter code. (A) Trigramin (7–33) shares 39.3% identity with human vWF precursor (1555–1581) in a 28 amino acid residue overlap. (B) Trigramin (1–43) also shows 35.7% identity with collagen α_1 (I) precursor (56–100) in a 42 amino acid residue overlap. (C) Trigramin (20–34) shows 53.3% identity with laminin B_1 (106–120) in a 15 amino acid residue overlap.

with prior reduction and the other without. Each sample was reisolated from the reaction mixture by reverse-phase HPLC and a portion (ca. 1 μg) subjected to amino acid analysis. The sample that had been reduced contained 12.0 mol of *S*-PE-Cys/mol of trigramin, while the nonreduced sample contained less than 0.2 mol/mol of protein, indicating that there were no free sulfhydryl groups in the molecule.

Primary Structure of Trigramin and Its Homology with Other Proteins. Figure 1 shows the whole sequence of trigramin established by mapping of proteolytic fragments. Trigramin appears to be a single-chain peptide consisting of 72 amino acids including an RGD sequence (positions 51–53) and six S–S bridges. The molecular weight of trigramin calculated from its amino acid sequence was 7507, and its calculated isoelectric point was at pH 5.61. The trigramin sequence was compared to sequences of known peptides by the Protein Identification Resource, National Biomedical Research Foundation, Georgetown University Medical Center, Washington, DC. The most relevant data are listed in Figure 2.

Trigramin shows 39% identity in a 28 amino acid overlap when compared with residues 1555–1581 of human vWF precursor; vWF also contains an RGD sequence (residues 1744–1746) near its carboxy-terminal end (Shelton-Inloes et al., 1986; Titani et al., 1986) (Figure 2). It is of interest that residues 1555–1581 of human vWF precursor are located in the putative GPIIb/IIIa binding domain of this protein (Titani et al., 1986). Trigramin also showed a 50% identity in a 15 amino acid fragment when compared with laminin, a specific basement membrane protein (Barlow et al., 1984). Furthermore, trigramin showed 36% identity in a 42 amino acid

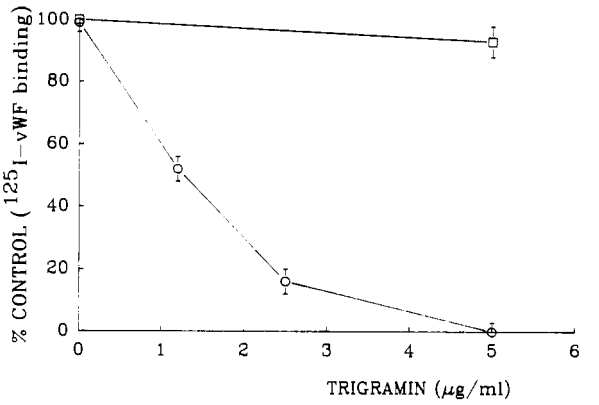

FIGURE 3: Effect of trigramin on ^{125}I-von Willebrand factor (vWF) binding to thrombin (0.5 unit/mL) stimulated platelets (○). The final concentration of human vWF was 5 μg/mL. The total specific vWF binding was 380 ± 55 ng/10^8 platelets in a control sample. Trigramin had no effect on ristocetin–induced ^{125}I-vWF binding (□). The specific vWF binding to ristocetin-stimulated platelets was 754 ± 140 ng/10^8 platelets in a control sample.

overlap when compared with collagen [α_1 (I) precursor (Chu et al., 1984)].

Inhibition of ^{125}I-vWF Binding to Platelets by Trigramin. In view of the existing homologies between vWF and trigramin and the observation that both proteins bind to the GPIIb/IIIa complex, we investigated the effect of trigramin on the binding of vWF to thrombin–stimulated human platelets. Thrombin (0.5 unit/ml) induced ^{125}I-vWF binding to platelets (379 ± 55 ng of vWF/10^8 platelets). As shown in Figure 3, trigramin (1–5 μg/mL; 0.11–0.55 μM) inhibited ^{125}I-vWF binding to thrombin-stimulated platelets. The IC_{50} of trigramin was about 0.11 μM. Reduced and pyridylethyleted trigramin was inactive even at concentrations as high as 2×10^{-6} M. Monoclonal antibodies against the GPIIb/IIIa complex, AP_2 (20 μg/mL) and A_2A_6 (10 μg/mL), almost completely inhibited ^{125}I-vWF binding (not shown). RGDS (50 μM) and tyrosylpentadecapeptide (100 μM) also showed complete inhibition.

Ristocetin (0.75 mg/mL) induced 754 ± 143 ng of ^{125}I-vWF binding/10^8 platelets. In three experiments, the amount of vWF bound to ristocetin-stimulated platelets in the presence of trigramin (5 μg/mL) (Figure 3) and AP_2 (20 μg/mL) amounted to 93 ± 5% and 86 ± 10% of control values, respectively. On the other hand, the amount of vWF bound to ristocetin-stimulated platelets in the presence of AP_1 monoclonal antibody against glycoprotein Ib amounted to 11 ± 1% of control values. These data strongly indicate that trigramin is specific in inhibiting the ^{125}I-vWF binding to glycoprotein IIb/IIIa but not to glycoprotein Ib.

Discussion

Trigramin, a peptide purified from the venom of *T. gramineus*, is a competitive inhibitor of fibrinogen binding to the platelet membrane GPIIb/IIIa complex (Huang et al., 1987). In this paper we established the primary structure of trigramin, indicating that it is an RGD-containing peptide and that it shares some homologous sequences with three adhesive proteins: human von Willebrand factor, collagen α_1 (I), and mouse laminin B_1. Trigramin is a single-chain polypeptide consisting of 72 amino acid residues and six disulfide bonds. Two striking similarities were observed between trigramin and human vWF: first, both molecules contained RGD in their carboxy-terminal domains (trigramin, positions 51–53; vWF precursor, positions 1744–1746). Second, both proteins shared 39% homologous sequences in a 28 amino acid fragment (trigramin, positions 7–33; vWF precursor, positions

1555–1581). The presence of five cysteines at analogous positions in this short sequence is a primary contributor to the extent of this homology. The 1555–1581 fragment is located in the GPIIb/IIIa binding domain of vWF (Titani et al., 1986). Both of these regions in the vWF precursor are present in the vWF molecule which circulates in plasma. Further investigations are needed to evaluate the biological significance of the homologies between the fragment of trigramin (position 7–33) and the fragment of vWF (position 1555–1581).

The disulfide bond structure of trigramin is of major importance for its ability to interfere with vWF binding to platelets, because reduction of trigramin markedly decreases its ability to interfere with vWF binding to platelets.

Our previous experiments on the binding of trigramin to platelets and on the inhibition of fibrinogen binding to platelets by trigramin (Huang et al., 1987) and our current data showing the inhibitory effect of trigramin on vWF binding to thrombin-stimulated platelets are compatible with the contention that the biological activity of trigramin is related to its binding to the GPIIb/IIIa complex. However, it is difficult to compare the potency of trigramin in inhibiting vWF and fibrinogen binding to GPIIb/IIIa due to the polymeric structure of vWF. It is noteworthy that trigramin does not seem to interact with GPIb, since it had no effect on ristocetin-stimulated platelets.

The RGD sequence is the cell-recognizing domain existing in a variety of adhesive proteins such as fibrinogen, vWF, fibronectin, and vitronectin (Ruoslahti & Pierschbacher, 1987). It is well established that RGD-containing peptides can inhibit both fibrinogen (Gartner & Bennett, 1985; Plow et al., 1985) and vWF binding (Williams & Gralnick, 1987) to the GPIIb/IIIa complex. A certain conformation of trigramin is a prerequisite for its binding activity toward this complex since reduction of the trigramin molecule results in a loss of activity to inhibit fibrinogen (Huang et al., 1987) and vWF binding to activated platelets (this study). This may explain why a large number of RGD-containing peptides are inactive in this regard (Ruoslahti & Pierschbacher, 1987) and why the K_i of RGDS is about 500–1000 times greater than that of trigramin (Huang et al., 1987).

Kloczewiak et al. (1984) localized one of the platelet recognition sites of fibrinogen to the carboxy-terminal dodecapeptide of the chain. However, trigramin contains no common sequence with this dodecapeptide. It is of interest that this dodecapeptide, which is also absent in vWF (Titani et al., 1986), inhibits binding of vWF (Williams & Gralnick, 1987) and binding of trigramin (Huang et al., 1987) to platelets.

It remains to be investigated whether homologous sequences between trigramin and collagen α_1 (I), or between trigramin and laminin, may contribute to the biological activity of these molecules although the homologous sequence between trigramin and laminin is different from the active site of laminin which mediates cell attachment, chemotaxis, and receptor binding. The peptide corresponding to this active site has been recently isolated by Graf et al. (1987). It is possible that the proposed homology between trigramin and these proteins is spurious and it is simply the result of finding many cysteine residues in a short peptide.

The primary structure of GPIIb and GPIIIa has been deduced from cDNA clones and indicates that GPIIb shares homology with the subunits of the vitronectin andd fibronectin receptors, whereas GPIIIa shares a common sequence with integrin (Fitzgerald et al., 1987; Poncz et al., 1987). Our recent studies in cooperation with Knudsen et al. (1988) demonstrated an inhibitory effect of trigramin on the adhesion of human melanoma cells and human fibroblasts to the substrate covered with fibrinogen and fibronectin. Compared on a molar basis to GRGDS, trigramin was approximately 500 times more active than this hexapeptide in inhibiting cell adhesion to fibronectin. Reduced and S-pyridylethylated trigramin had no effect in this system. These data suggest a possible interaction of trigramin with vitronectin and fibronectin receptors present on melanoma cells and fibroblasts.

In conclusion, we determined the primary structure of trigramin, a low molecular weight peptide that binds to the platelet membrane GPIIb/IIIa complex with a high affinity. Trigramin is an RGD-containing peptide with six disulfide bonds. At concentrations inhibitory for fibrinogen binding to platelets, trigramin also inhibited vWF binding to GPIIb/IIIa. The disulfide bridges of trigramin may play an important role in maintaining its conformation and expressing its biological activity. The molecular basis for the interaction of trigramin with the GPIIb/IIIa complex is an important issue that needs to be explored.

Registry No. Trigramin, 111019-84-2; trigramin (*T. gramineus*, reduced), 117939-38-5; von Willebrand factor, 109319-16-6.

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Subunit Structure of Thrombin-Activated Porcine Factor VIII†

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ABSTRACT: Factor VIII (fVIII) is synthesized as a single chain having a domainal sequence A1-A2-B-A3-C1-C2. Analysis of the proteolyic cleavage of fVIII by thrombin by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) identifies three fragments designated $fVIII_{A1}$, $fVIII_{A2}$, and $fVIII_{A3\text{-}C1\text{-}C2}$ with fragment(s) derived from the B domain being difficult to visualize. The appearance of these fragments is associated with the development of coagulant activity, but the activity is labile without further apparent proteolysis. In this study, porcine fVIII was reacted with thrombin until peak coagulant activity was obtained and then subjected to cation-exchange (Mono S) high-pressure liquid chromatography. Coagulant activity was recovered in a single peak that contained all three fragments and was stable for weeks at 20 °C in 0.65 M NaCl/0.01 M His-HCl/0.005 M $CaCl_2$ at pH 6.0. Analytical ultracentrifugation of activated fVIII was done to test whether all three fragments were associated. The apparent molecular weight of activated fVIII from equilibrium sedimentation increased from 148 000 to 161 000 as the loading concentration was increased from 0.06 to 0.16 mg/mL. This agrees well with the summed apparent molecular weights of $fVIII_{A1}$, $fVIII_{A2}$, and $fVIII_{A3\text{-}C1\text{-}C2}$ calculated from SDS–PAGE analysis (148 000) or from the amino acid sequence of human fVIII (159 000). This establishes the major species in the preparation as a $fVIII_{A1/A2/A3\text{-}C1\text{-}C2}$ heterotrimer and additionally indicates either weak self-association of the trimer and/or incomplete association of the individual subunits to form the trimer. Velocity sedimentation of activated fVIII revealed a single boundary ($s^0_{20,w}$ = 7.2 S). From the combined velocity and equilibrium sedimentation data, a frictional coefficient ratio of 1.39 was calculated, indicating that activated fVIII is moderately asymmetrical.

Factor VIII (fVIII),[1] the plasma protein that is decreased or absent in patients with hemophilia A, has been the subject of extensive biochemical and clinical investigation for over 50 years. It circulates tightly bound in a noncovalent complex with von Willebrand factor (vWf). Recent advances have

Table I: Yield of $FVIIIa_{IIa}$ from Mono S Chromatography[a]

detergent[b]	expts	fractional yield[c]	specific activity[d]
yes	6	0.57 ± 0.10[e]	7000 ± 800[e]
no	2	0.58	8100

[a] The yield ranged from 0.5 to 2.2 nmol (0.07–0.33 mg). [b] 0.01% Tween 80. [c] Mole of $fVIIIa_{IIa}$ recovered per mole of fVIII in activation mixture. [d] $FVIIIa_{IIa}$ coagulant activity (units per nanomole). [e] Mean ± SD.

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