A Novel Peptide Motif for Platelet Fibrinogen Receptor Recognition*

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To develop a specific antagonist of platelet αIIbβ3 using small linear peptides, we synthesized a series of hexapeptides that did not have an Arg-Gly-Asp (RGD) sequence and examined their anti-platelet activity and their specificity for αIIbβ3. We found a novel motif sequence, Pro-X1-X2-X3-Asp-X4, where X1 to X4 were all L-form α-amino acids, which specifically inhibited aggregation of human platelets at submicromolar concentrations. The Pro residue at the N terminus was essential to the anti-platelet activity, and the acetylation of the imino group of this residue also resulted in the complete loss of the activity. The results of the binding assay using purified human platelet αIIbβ3 and placental αvβ3 and those of the cell adhesion assay suggest that this motif peptide is highly specific for platelet αIIbβ3 among other integrins. Flow cytometric studies using an fluorescein isothiocyanate-labeled RGD peptide showed that this motif peptide inhibited the binding of an RGD peptide to activated platelets, suggesting that it has the same inhibitory mode as RGD peptides. Conformational analysis of this motif peptide and an RGD-containing peptide suggests that the imino group of the Pro residue may substitute for the role of the guanidino group of the Arg residue of the RGD sequence.

Integrins are heterodimeric cell surface receptor molecules and are thought to be particularly important mediators of cell adhesion, cell migration, and adhesion-dependent intracellular signaling. Until now, more than 20 integrin receptors have been identified, and many of them recognize the Arg-Gly-Asp sequence, which are highly specific for platelet αIIbβ3. Several peptides without the RGD sequence, which do not have the RGD sequence, are potent antagonists of this integrin (21). Another example is barbourin, which is a member of the disintegrin family and shows high specificity for this integrin (22). The aim of our study is to develop a potent and highly specific αIIbβ3 antagonist of small linear peptides (less than six residues) consisting of natural amino acids. We found a novel motif sequence of a hexapeptide. One of the motif peptides, Pro-Ser-Hyp-Gly-Asp-Trp (Hyp representing 4-hydroxy-L-proline) (NSL-9511), inhibited platelet aggregation at a submicromolar concentration, although it did not affect adhesion of human endothelial cells to immobilized vitronectin and fibronectin. The results of the solid phase binding assay suggest that this peptide is a specific antagonist of platelet αIIbβ3. NSL-9511

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The abbreviations used are: RGD, Arg-Gly-Asp; FITC, fluorescein isothiocyanate; PRP, platelet-rich plasma; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay.
also exhibited antithrombotic activity in an in vivo thrombosis model.

EXPERIMENTAL PROCEDURES

**In Vitro Platelet Aggregation**—Platelet aggregation studies were performed in platelet-rich plasma (PRP) obtained from various animal species, including humans. Blood was drawn into plastic syringes containing 1% volume of 3.8% trisodium citrate. PRP was prepared by centrifugation of citrated whole blood at 160 × g for 15 min at room temperature. PRP was removed, and the platelet count was determined. Platelet-poor plasma was obtained by centrifugation of the remaining blood at 2000 × g for 15 min. Saline or peptide solution of various concentrations was added to PRP at 37 °C 1 min prior to the initiation of platelet aggregation. Platelet aggregation was initiated with 5 μg/ml collagen, and the aggregation was measured in an aggregometer (NBS Hematrac-601, Nikoh Bioscience Co., Ltd., Tokyo) as an increase in light transmission. Platelet aggregation is presented as the percentage of inhibition of the rate of platelet aggregation compared with control samples, and the IC50 values were calculated from dose-inhibition curves.

**Preparation of Integrin Receptors**—Vitronectin receptor (αvβ3) was purified according to the procedures described elsewhere (17, 23) with some modifications. Briefly, human platelets were collected in a plastic bag and extracted with buffer containing 100 mM octyl glucoside. The extract was centrifuged, and the supernatant was applied to a Sepharose 4B column and then applied to a GRGDSPK-Sepharose affinity column. The column was washed with five bed volumes of the same solution at room temperature with 10 mM EDTA and 50 mM octyl glucoside. Finally, Ca2+ was added to the eluted fraction so that the final concentration of the free Ca2+ was 2 mM.

Platelet fibrinogen receptor (αIIbβ3) was purified from outdated human platelets using a procedure almost identical to that for αvβ3. Briefly, the platelets were washed three times with TBS containing 1 mM CaCl2 and 100 mM octyl glucoside and then resuspended in TBS containing 100 mM octyl glucoside, 1 mM MnCl2, 1 mM MgCl2, and 0.1 mM phenylmethylsulfonyl fluoride. After the centrifugation at 30,000 × g, the supernatant fraction was applied to a Sepharose 2B column, and the flow-through fraction was then applied to a GRGDSPK-Sepharose affinity column. Bound fibrinogen receptor was eluted with TBS containing 50 mM octyl glucoside and 5 mM GRGDSP peptide. The eluted fraction was analyzed by MALDITOF mass spectrometry. After the elution, the peptide was purified from the supernatant by reversed-phase HPLC using a C18 preparative column. The purity of the peptides was examined by MALDI-TOF mass spectrometry.

**Solid Phase Binding Assay**—The inhibitory effects of the peptides on integrin-ligand interactions were evaluated by using a competitive enzyme-linked immunosorbent assay (ELISA) (17). Briefly, for vitronectin receptor, 96 -well microtiter plates were coated with vitronectin (purified from outdated human plasma, Iwaki Glass Co., Ltd., Tokyo) or fibrinogen (23). EILDVPST peptide (Funakoshi, Tokyo, Japan), originating from the CS-1 site of fibrinogen (23), was used to examine the contribution of the CS-1 site of the peptide to cell adhesion to fibrinogen.

**Guinea Pig Arteriovenous Shunt Model**—Male Hartley guinea pigs weighing 230–320 g were anesthetized with pentobarbital (35 mg/kg, intraperitoneal), and an intratracheal cannula was inserted through the incision. To maintain a moderate anesthesia during the experiment, additional pentobarbital administration (intraperitoneal) was done if necessary. The polyethylene tubes filled with saline containing 100 units/ml heparin were inserted into the left jugular vein and right carotid artery, and these catheters were connected on a Micron YM 30 filter. Purified human αIIbβ3, purchased from Enzyme Research Laboratories, Inc., was also used.

**Cell Adhesion Assay**—The adhesion assay was performed in 24-well tissue culture plates. Each well was precoated with 30 μg/ml fibronectin (purified from human plasma, Iwaki Glass Co., Ltd., Tokyo) or 3 μg/ml vitronectin (purified from bovine plasma, Iwaki Glass Co., Ltd., Tokyo). After 1 h at 4 °C, the platelet suspension was centrifuged at 1200 × g for 1 min prior to the initiation of the adhesion assay. The infranatant was aspirated, and the platelet count was determined. Platelet aggregation was initiated with 5 μg/ml collagen, and the aggregation was measured in an aggregometer (NBS Hematrac-601, Nikoh Bioscience Co., Ltd., Tokyo) as an increase in light transmission. Platelet aggregation is presented as the percentage of inhibition of the rate of platelet aggregation compared with control samples, and the IC50 values were calculated from dose-inhibition curves.

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In our preliminary studies using flow cytometry, we have found that NSL-9511 inhibited the binding of fibrinogen to ADP-activated platelets, suggesting that the anti-platelet activity of NSL-9511 results from the blockade of fibrinogen binding to its receptor on the platelet surface. To clarify the mechanism underlying the anti-platelet activity further, we performed a competitive ELISA in which the binding of purified human platelet fibrinogen receptor, αIIbβ3, to immobilized human fibrinogen was detected by specific antibody. αvβ3 ELISA was also performed using αvβ3 purified from human placenta to examine the binding specificity of NSL-9511. As Fig. 1a shows, 100 μM NSL-9511 almost completely inhibited the binding of αIIbβ3 to fibrinogen, whereas it did not inhibit the binding of αvβ3 to vitronectin. An RGD-containing peptide, GRGDS, at the same concentration did inhibit both the binding of fibrinogen to αIIbβ3 and that of vitronectin to αvβ3 completely. These results suggest that NSL-9511 is highly specific for platelet αIIbβ3. Anti-αv monoclonal antibody and anti-αIIbβ3, both of which have been reported as binding-blocking antibody, completely inhibited the binding of vitronectin to αvβ3 and fibrinogen to αIIbβ3, respectively. As Fig. 1b shows, NSL-9511 inhibited the binding of fibrinogen to αIIbβ3 in a dose-dependent manner with an IC50 value of 70 nM. On the other hand, this compound hardly inhibited the binding of vitronectin to αvβ3 and the percentage of inhibition was only 23% at the highest dose (0.6 mM).

**Effects on Binding of RGD Peptide to Activated Platelets**—Although NSL-9511 is a potent antagonist of αIIbβ3, it does not possess the RGD sequence. To determine whether NSL-9511 and the RGD peptide are mutually exclusive upon binding to αIIbβ3, we synthesized FITC-labeled RGD peptide and examined the effects of NSL-9511 on the binding of this RGD peptide to activated platelets. As Fig. 2 shows, activation of washed platelets with ADP increased the mean fluorescence when the platelets were incubated with 100 μM FITC-WSRGDW. This increment in mean fluorescence was inhibited by the addition of excess unlabeled RGD peptide (1 mM WSRGDW) (data not shown), suggesting that it resulted from the specific binding of FITC-labeled WSRGDW to platelet integrins in an activation-dependent manner. An addition of 10 μM NSL-9511 completely inhibited the increase in the mean fluorescence. These results suggest that NSL-9511 and the RGD peptide share the same binding site on αIIbβ3 or that their binding sites are very close.

**Effects on Cell Adhesion to Vitronectin and Fibronectin**—The binding specificity of NSL-9511 was examined in a cell adhesion assay, in which ECV304 cells, originating from human umbilical vein endothelial cells, adhered to immobilized vitronectin and fibronectin via integrin receptors. As shown in Fig. 3a, the adhesion of ECV 304 cells to immobilized human fibronectin was inhibited by the GRGDS peptide, and almost complete inhibition was observed at 1000 μM. The EILDVPST peptide, which is the sequence within the CS-1 site of fibronectin and is considered to be another sequence responsible for the adhesion of various cell types to fibronectin (28), did not show such an inhibitory activity up to 100 μM under this assay condition. These results suggest that the adhesion of ECV 304

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### Table I

**Peptide Motif for Platelet Fibrinogen Receptor Recognition**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Motif for Platelet Fibrinogen Receptor Recognition</th>
</tr>
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<tbody>
<tr>
<td>PSRGDW</td>
<td>GRG</td>
</tr>
<tr>
<td>SRGDW</td>
<td>GRG</td>
</tr>
<tr>
<td>PS-Nva-GDW&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GRG</td>
</tr>
<tr>
<td>GRDPS</td>
<td>GRG</td>
</tr>
<tr>
<td>PS-Hyp-GDW&lt;sup&gt;b&lt;/sup&gt; (NSL-9511)</td>
<td>GRG</td>
</tr>
<tr>
<td>CH&lt;sub&gt;3&lt;/sub&gt;CO-PSPGDW&lt;sup&gt;c&lt;/sup&gt;</td>
<td>GRG</td>
</tr>
<tr>
<td>GRGDS</td>
<td>GRG</td>
</tr>
</tbody>
</table>

<sup>a</sup> Nva, L-norvaline.
<sup>b</sup> Hyp, 4-hydroxy-L-proline.
<sup>c</sup> CH<sub>3</sub>CO-P, N-acetyl-L-proline.

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### Table II

**Inhibition of platelet aggregation by NSL-9511 in PRP from different species**

<table>
<thead>
<tr>
<th>Species</th>
<th>Platelet aggregation (IC&lt;sub&gt;50&lt;/sub&gt;) μM</th>
</tr>
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<tbody>
<tr>
<td>Human</td>
<td>0.39</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>0.87</td>
</tr>
<tr>
<td>Mouse</td>
<td>40.0</td>
</tr>
<tr>
<td>Rat</td>
<td>1000</td>
</tr>
</tbody>
</table>

Collagen (5 μg/ml)-induced platelet aggregation using PRP from different species is shown.

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<sup>a</sup> Nva, L-norvaline.
<sup>b</sup> Hyp, 4-hydroxy-L-proline.
<sup>c</sup> CH<sub>3</sub>CO-P, N-acetyl-L-proline.
translation of blood in this shunt results in a platelet-rich thrombus
in vivo formation at a dose of 100 mg/kg/h in this model. Although the antithrombotic effect of heparin continued for at least 60 min after the termination of its infusion, the effect of NSL-9511 disappeared quickly, and the thrombus formation was examined for another 60 min (Fig. 4). Although the antithrombotic effect of heparin continued for at least 60 min after the termination of its infusion, the effect of NSL-9511 disappeared quickly, and the thrombus formation was examined for another 60 min (Fig. 4). Although the antithrombotic effect of heparin continued for at least 60 min after the termination of its infusion, the effect of NSL-9511 disappeared quickly, and the thrombus formation was examined for another 60 min (Fig. 4). Although the antithrombotic effect of heparin continued for at least 60 min after the termination of its infusion, the effect of NSL-9511 disappeared quickly, and the thrombus formation was examined for another 60 min (Fig. 4).

**DISCUSSION**

We have been developing specific antagonists of integrin receptors such as αIIbβ3, αvβ3, or α5β1 using small linear peptides as the basic structure, because they are, in general, easy to synthesize by standard solid phase methods, and we can obtain much information about the structure-activity relationship by changing the constituent amino acids. In this study, we have found a series of potent and highly specific antagonists of platelet αIIbβ3 integrin that are linear hexapeptides without the RGD sequence. Although several peptide αIIbβ3 antagonists with high specificity for this integrin have already been reported, such as fibrinogen γ-chain C-terminal decapetide (19, 20), a tick saliva protein (21), barbourin (22), and synthetic cyclic peptides (16–18), this is the first report of small linear ones using L-form natural amino acids. Scarborough et al. (22) have reported that barbourin, a member of the disintegrin family of snake venom proteins, inhibits the binding of fibrinogen to αIIbβ3 but does not block vitronectin binding to αvβ3. Barbourin has a KGD sequence rather than RGD; therefore,
the Lys residue is thought to be critical for the binding specificity. These results suggest that for potent antagonism toward integrins that recognize a RGD sequence such as \( \alpha_{IIb} \beta_3 \), \( \alpha_v \beta_3 \), or \( \alpha_5 \beta_1 \), two interacting sites, an acidic moiety and a basic moiety, are necessary, and the substitution for a guanidino group of the Arg residue by the other basic groups can produce the binding specificity. Our results described here also demonstrate that the guanidino group of the Arg residue within the RGD sequence is not unique to the antagonistic activity toward \( \alpha_{IIb} \beta_3 \), that the imino group of proline may displace the role of the guanidino group, and that the introduction of the Pro residue to the N terminus of the peptide results in more potent activity and binding specificity.

Now we are studying the close structure-activity relationships around this peptide. Because the N-terminal Pro residue and the Asp residue at the fifth position are essential to the activity, the basic structure of the peptides can be summarized to have the following motif, Pro-\( X_1 \)-Asp-\( X_4 \). The results of the preliminary experiments suggest that 1) a small amino acid such as Ser, Ala, or Gly is preferable at the \( X_1 \) position, 2) \( X_2 \) may be any amino acid, 3) \( X_3 \) must be a small amino acid with a residue such as Gly, or a cyclic amino acid such as Pro, and 4) \( X_4 \) prefers an amino acid with an aromatic side chain.

We are going to discuss the structure-activity relationship of this peptide in detail elsewhere.

One of the motif peptides, NSL-9511, inhibited the binding of fibrinogen to purified human platelet \( \alpha_{IIb} \beta_3 \) in a dose-dependent manner with an IC\(_{50}\) of 70 nM (Fig. 1). Because NSL-9511 also inhibited in vitro platelet aggregation at submicromolar concentrations (IC\(_{50}\) = 390 nM), the anti-platelet activity of NSL-9511 can be explained by the blockage of the fibrinogen binding to \( \alpha_{IIb} \beta_3 \). Further, in this binding assay, the concentration of fibrinogen in the incubation medium did not greatly affect the IC\(_{50}\) value of NSL-9511 (data not shown). These results suggest that NSL-9511 inhibits fibrinogen binding, not by interacting with fibrinogen molecules but by binding to \( \alpha_{IIb} \beta_3 \) integrin as an RGD peptide does. Therefore, we concluded that NSL-9511 is an \( \alpha_{IIb} \beta_3 \) antagonist.

Because NSL-9511 does not have an RGD sequence and is a much more potent antagonist of \( \alpha_{IIb} \beta_3 \), we then examined whether the binding mode of NSL-9511 and an RGD peptide is the same. The results of the experiment showing that NSL-9511 dose-dependently inhibited the binding of an FITC-labeled RGD peptide to activated platelets (Fig. 2) suggest that NSL-9511 and the RGD peptide share the same binding site on \( \alpha_{IIb} \beta_3 \) or that the binding site of NSL-9511 lies so close to the...
FIG. 5. The conformational analysis of PSRGDW. The conformational analysis was conducted on Macro Model, version 3.5. Fifty thousand conformers were generated by the Monte Carlo method and minimized with a modified Amber* Force field in vacuum to obtain 2200 conformers. The distance between the guanidino group of the Arg residue and the carboxy group of the Asp residue (d1) and the distance between the imino group of the Pro residue and the carboxy group of the Asp residue (d2) were calculated. Conformers with shorter d1 or d2 (<10 Å) were eliminated, based on Kessler’s active conformation. The electrostatic cut-off distance was set to 5 Å, and the force field was modified in order to not evaluate hydrogen bonds. The largely overlapped area of d1 and d2 distribution suggests that the relative arrangement between the Pro residue and the Asp residue is similar to that of the Arg residue.

RGD-binding site that NSL-9511 and an RGD peptide are mutually exclusive upon binding to αIIbβ3.

These findings, that NSL-9511 is an αIIbβ3 antagonist and possibly has the same binding mode as an RGD peptide and that the N-acetylation of the imino group of the Pro residue results in the complete loss of the anti-platelet activity, lead us to the idea that the imino group of the Pro residue at the N terminus replaces in a complementary manner the function of the guanidino group of the Arg residue within an RGD sequence as a basic moiety essential to the activity. This idea is supported by the conformational analysis of a PSRGDW peptide, which has both the imino group of the Pro residue and the guanidino group of the Arg residue. The spatial distance between the carboxyl group of the Asp residue and the guanidino group of the Arg residue (d1) of stable conformers and the spatial distance between the carboxyl group and the imino group of the Pro residue (d2) of stable conformers were calculated (Fig. 5). The largely overlapped area of d1 and d2 distribution suggests that the relative arrangement between the Pro residue and the Asp residue is similar to that of the Arg residue, indicating that the imino group of the Pro residue can occupy the same conformational space as the guanidino group of the Arg residue.

To examine the binding specificities of the motif peptide, we performed the binding assay using purified human placental αvβ3 and vitronectin and the cell adhesion assay, in which ECV 304 adhered to immobilized fibronectin or vitronectin. In the αvβ3-vitronectin binding assay, both an anti-αvβ3 monoclonal antibody and 100 μg GRGDS completely inhibited the binding, suggesting that the binding was mediated by αvβ3 and the RGD sequence of vitronectin. The inhibition of the binding of vitronectin to αvβ3 by NSL-9511 was very weak at its highest concentration (Fig. 1b), suggesting that NSL-9511 is highly specific for αIIbβ3. The results of the cell adhesion assay (Fig. 3) also show that NSL-9511 did not inhibit cell adhesion via other integrins such as αvβ3 or α5β1. ECV304 is a cell line established from human umbilical vein endothelial cells, and we have confirmed the expression of αvβ3 and α5β1 on ECV304 using monoclonal antibodies by flow cytometry (data not shown). Adhesion of ECV304 to vitronectin is thought to be largely mediated by αvβ3 because the monoclonal antibody to the αvβ3 complex inhibited the adhesion. However, we could not show the dependence of α5β1 on the adhesion of ECV304 to fibronectin, because an anti-α5β1 antibody that has been reported to block the binding of fibronectin to α5β1 is not available. These results suggest that NSL-9511 is an antagonist highly specific for platelet αIIbβ3 but not for other integrins such as αvβ3 or α5β1. There are several possible explanations for this high specificity of NSL-9511 for αIIbβ3. First, it is possible that integrins other than αIIbβ3 prefer a guanidino group as a basic interaction site upon binding with the ligand, while both a guanidino group and secondary amines such as an imino group of the Pro residue are preferable as a basic moiety upon ligand binding to αIIbβ3. It is also possible that the active spatial structure for αvβ3 or α5β1 integrin is quite different from that for αIIbβ3 and that the NSL-9511 cannot conform to this structure, while the RGD peptide can.

NSL-9511 also exhibited the anti-platelet activity in vivo, when infused intravenously (Fig. 4). Infusion of NSL-9511 at a rate of 10 mg/kg/h almost completely inhibited the platelet-rich thrombus formation around the thread, and this antithrombotic activity quickly wore off after the termination of the infusion. Peptides, including NSL-9511 are rapidly degraded in guinea pig plasma at 37 °C by the action of aminopeptidases (data not shown); therefore, this quick disappearance of antithrombotic activity would be due to the decrease in plasma concentration of NSL-9511 caused by the enzymatic degradation. The same is true for an RGDS peptide, which did not show any antithrombotic activity in this shunt model. Substantial increases in bleeding time have been an undesirable characteristic exhibited by many αIIbβ3 antagonists (34) including an anti-αIIbβ3 monoclonal antibody, 7E3 (35, 36), and this is one of the limitation factors in the use of an αIIbβ3 antagonist as an antithrombotic agent. Therefore, NSL-9511, which exhibits potent antithrombotic activity in vivo during the infusion, although this activity wears off quickly and the prolongation of bleeding time is not as serious, has a potential for clinical uses, especially as an antithrombotic drug at an acute phase.

Another interest of ours is the possibility that these motif peptides can be produced endogenously. Although many of the human proteins include the sequences homologous to this motif sequence, they do not lie at the N terminus, and the imino group of the Pro residue is not free. To exert anti-platelet activity, these proteins need to be processed so as to expose the Pro residue at a new N terminus. Aminopeptidase P, which has been reported to exist in human cells such as platelets (37), and some virus proteases such as HIV-1 protease (38) are candidates for catalyzing such a reaction.

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