Structural and Interaction Analysis of Glycoprotein VI-binding Peptide Selected from a Phage Display Library*§

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Glycoprotein VI (GPVI) is a major collagen receptor on the platelet surface that recognizes the glycine-proline-hydroxyproline (GPO) sequence in the collagen molecule and plays a crucial role in thrombus formation. Inhibitors that block the interaction of GPVI with collagen have potential for use as antithrombotic drugs. For low molecular weight drug design for GPVI, it is essential to obtain precise structural and interaction information about GPVI-binding ligands. However, experimentally obtained structural and interaction information of small ligands, such as peptides, in the GPVI-bound state has not been reported. In this study, by screening a phage-displayed peptide library, we discovered a novel peptide ligand (pep-10L; YSDTDLWLYFSTS) without any similarities to the sequence of collagen that inhibits GPVI-GPO binding. Systematic Ala scanning in surface plasmon resonance experiments and a saturation transfer difference NMR experiment revealed that Trp^6, Leu^7, Phe^9, and Ser^10 residues in the pep-10L peptide interacted with GPVI. Furthermore, the GPVI-bound conformation of the pep-10L peptide was determined using transferred nuclear Overhauser effect analysis. The obtained structure has revealed that the central part of pep-10L (Asp^5–Phe^9) has a helical conformation, the side chains of Trp^6, Leu^7, and Phe^9 form a hydrophobic side in the helix, and the Tyr^8 side chain faces the opposite direction from the hydrophobic side. Computational docking prediction has shown that the hydrophobic side of pep-10L sticks in the hydrophobic groove on the GPVI surface, which corresponds to the putative collagen-related peptide binding groove. These data could enable the structure-guided development of a small molecule GPVI antagonist.

Glycoprotein VI (GPVI)^3 is a major collagen receptor on the platelet surface that plays a crucial role in collagen-induced platelet activation, thrombus formation (1–3), and acute coronary syndrome (4). The platelets of GPVI^null mice fail to aggregate in response to collagen and lack thrombus formation, but the mice do not show a severe bleeding tendency (5). In clinical studies, GPVI-deficient patients show only a mild bleeding tendency despite the fact that their platelets lack collagen-induced aggregation (6–8). In addition, the Fab fragment of a monoclonal antibody, OM4, effectively inhibits thrombosis in rats without prolonging bleeding (9). These observations suggest that inhibitors that block the interaction of GPVI with collagen could be effective antithrombotic drugs without severe side effects. For low molecular weight drug design, it is essential to elucidate the ligand recognition mechanism and to obtain precise structural and interaction information for GPVI-binding ligands.

GPVI recognizes the glycine-proline-hydroxyproline (GPO) sequence in the collagen molecule (10). In addition to collagen, synthetic collagen-related peptide (CRP), which contains 10 GPO repeats, is also a specific agonist for GPVI and is commonly used to study GPVI function (11). Recently, the minimum recognition motif for GPVI has been identified as GPO or GPOGPO (12), and it is suggested that the inhibition of GPVI binding to this motif is important for the inhibitory effect of platelet aggregation (12). Other studies, using blocking phage antibodies, site-directed mutagenesis, and crystal structure-based computational docking prediction, have shown the possibility that GPVI has multiple binding sites (11, 13, 14). However, experimentally obtained structural and interaction information of a small ligand, such as a peptide, in the GPVI bound-state has not been reported.

Recently, we established a rapid and easy preparation method for stable-isotope labeled peptides, which are directly obtained from a bacteriophage library (15). NMR is a versatile and useful technique for structural determinations and also for analyses of the interactions of peptides bound to target molecules with various affinities. The structural and interaction information obtained by NMR analyses provide insight into improving the affinity of peptides and/or the design of the small molecules that bind to the target protein.

In this study, novel peptide ligands that inhibit GPVI-GPO binding were found through screening a phage-displayed peptide library, and NMR analyses were performed. The amino acid residues interacting with GPVI were found by systematic Ala scanning of the peptide with surface plasmon resonance...
Glycoprotein VI-binding Peptide Analysis

Preparation of Recombinant GPVI-Fc2—Recombinant human GPVI protein (residues Gln21–Phe234) was expressed as described below from backbone peptide, according to a previous report (17). The GPVI peptide sequence: (GPP)8GPO(GPP)4GPCCGGG was dissolved in 200 mM Tris-HCl (pH 8.0) containing 200 mM NaCl and 1 mM EDTA (1 mg/ml); then, 9 mM GSSG and 0.9 mM GSH were added. After incubation for 5 days at 20 °C, the solution was dialyzed with 5 mM phosphate buffer (pH 7.4) containing 150 mM NaCl. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Shimadzu, Kyoto, Japan) was used to confirm the molecular weights of the GPVI-1 homotrimer. The GPO-1 homotrimer peptide was biotinylated with 0.1 M biotin (Pierce) after dialysis with 5 mM phosphate buffer (pH 7.4); then, 9 mM GSSG and 0.9 mM GSH were added. After incubation for 5 days at 20 °C, the solution was dialyzed with 5 mM phosphate buffer (pH 7.4) containing 150 mM NaCl. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Shimadzu, Kyoto, Japan) was used to confirm the molecular weights of the GPVI-1 homotrimer. The GPO-1 homotrimer peptide was biotinylated using an EZ-Link Sulfo-NHS-Biotinylation Kit (Pierce) according to the manufacturer’s instructions.

Preparation of Recombinant GPVI-Fc2—Recombinant dimeric GPVI-Fc (GPVI-Fc2) were selected in a previous report (17). The GPO-1 peptide sequence: (GPP)8GPO(GPP)4GPCCGGG was dissolved in 200 mM Tris-HCl (pH 8.0) containing 200 mM NaCl and 1 mM EDTA (1 mg/ml); then, 9 mM GSSG and 0.9 mM GSH were added. After incubation for 5 days at 20 °C, the solution was dialyzed with 5 mM phosphate buffer (pH 7.4) containing 150 mM NaCl. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Shimadzu, Kyoto, Japan) was used to confirm the molecular weights of the GPVI-1 homotrimer. The GPO-1 homotrimer peptide was biotinylated using an EZ-Link Sulfo-NHS-Biotinylation Kit (Pierce) according to the manufacturer’s instructions.

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**Glycoprotein VI-binding Peptide Analysis**

**SPR Analysis**—SPR analysis was performed at 25 °C using a BIacore 3000 instrument (Amersham Biosciences) and HBS-EP buffer (10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 3 mM EDTA, and 0.005% v/v surfactant P20). Human type I collagen (Chemicon, Temecula, CA) and CRP (sequence: GKO(GPO)10KOG) were immobilized on a research grade CM5 sensor chip (Amersham Biosciences) using an Amine Coupling Kit (Amersham Biosciences) according to the manufacturer’s instructions. The biotinylated GPO-1 homotrimer peptide was immobilized on an SA sensor chip (Amersham Biosciences). GPVI-Fc2 in HBS-EP buffer was injected over the ligand-immobilized surface at a flow rate of 20 μl/min, and the maximum response was recorded. For competitive experiments, GPVI-Fc2 (1 μM for CRP, 12 μM for collagen, and 5 μM for GPO-1) and peptide (0–2000 μM) were incubated before injection. Based upon the dose-dependent inhibition curve, IC_{50} values could be determined and the inhibition constants (K_{i}) were calculated by the Cheng-Poroshoff equation: 
\[ K_{i} = IC_{50}/(1 + [L]/K_{D}) \]
where [L] is GPVI-Fc2 concentration and K_{D} is the dissociation constant of the interaction between GPVI-Fc2 and each immobilized ligand.

Each sample was also analyzed using a flow cell without ligands for a control. The sensorgram of the ligand immobilized surface was subtracted by that of the control surface.

**Preparation of Labeled Peptide**—Tandem repeat inserts were prepared by annealing (70 °C for 5 min and then cooled to 25 °C over a 3-h period) and ligation of oligonucleotides encoding the pep-10L peptide sequences 5'-G-TACTCTGACACCGACTG-3' and 5'-G-GTGAAGAGA-GTACGACCGCTCGTGATGAT-3'. The resulting tandem repeat inserts were ligated into the pET31b(+)-containing plasmid (AlwNI-digested and dephosphorylated) containing a gene encoding ketosteroid isomerase and a His tag, which facilitate the purification of target peptides (Novagen, Madison, WI). The plasmid was transformed into *E. coli* BLR(DE3)pLysS (a *recA*− derivative of BL21); as anticipated, this resulted in a series of products with a varying number of pep-10L insertions. The insertion of pep-10L and its tandem repeats was confirmed by DNA sequence analysis (Applied Biosystems), and a plasmid containing four copies of the pep-10L coding sequence was selected.

The cells were grown in 13C/15N CHL medium (Chlorella Industry, Tokyo, Japan) containing carbenicillin (50 μg/ml) and chloramphenicol (34 μg/ml) at 37 °C until the A_{600} = 0.6 and were then induced for 4 h at 37 °C with isopropyl-1-thio-β-d-galactopyranoside (1 mM). The cells were harvested, and then the inclusion body was prepared. The inclusion bodies were dissolved in 6 M guanidine-HCl, and the ketosteroid isomerase/pep-10L peptide/His tag fusion protein was purified using nickel-nitritoltriacetic acid-agarose (Qiagen) affinity chromatography in the denatured condition. After imidazole was removed from the fraction containing the ketosteroid isomerase/pep-10L peptide/His tag fusion protein using a PD-10 desalting column (Amersham Biosciences), 1 M HCl (final concentration of 0.1 M), and 400 mg/ml CNBr (Wako) in acetonitrile (final concentration of 20 mg/ml) were added to the solution. The solution was stirred overnight at room temperature. The CNBr-cleaved peptide mixture was centrifuged (9000 × g, 5 min), and the supernatant was roughly separated using C_{18} Sep-Pak® (Waters). The 30% acetonitrile fractions were lyophilized, diluted with water, and further purified by reversed-phase high-performance liquid chromatography using a YMC-Pack PROTEIN-RP (YMC, Kyoto, Japan) with a gradient of H_{2}O/ acetonitrile containing 0.1% trifluoroacetic acid. The molecular weight of the labeled pep-10L peptide was confirmed by MALDI-TOF mass spectrometry (Shimadzu).

**NMR Spectroscopy**—The assignments of the backbone (13C_{α}, 13C_{β}, and 13C_{γ}) and 13C_{ν} resonances of pep-10L were achieved by using HNCACB (19) and CBCA(CO)NH (20). The spectra of the pep-10L peptide at 407 μM were recorded at 298 K in 20 mM phosphate buffer (pH 6.5), prepared in 95% H_{2}O and 5% D_{2}O. The assignments of side-chain 1H and 13C resonances were obtained from the combined use of HCCH-COSY (21) and HCCH-TOCSY (22), with the knowledge of the assigned 13C_{α} and 13C_{γ} resonances. The spectra of the pep-10L peptide at 375 μM were recorded at 298 K in 20 mM phosphate buffer (pH 6.5), prepared in 99.9% D_{2}O. All spectra were processed by NMRPipe (23), and the software Sparky was used to assist in data analyses.

For the two-dimensional STD-HSQC experiments, 130 μg of lyophilized 13C/15N-pep-10L peptide was dissolved in 275 μl of 99.9% D_{2}O/50 mM NaCl/20 mM phosphate buffer (pH 6.5) containing 405 μg of GPVI-Fc2 (16). The final concentrations of the peptide and GPVI-Fc2 were 300 μM and 12.3 μM, respectively. The two-dimensional STD-HSQC experiments were performed at 288 K on a Bruker Avance 800-MHz spectrometer equipped with a cryo-cooled triple-resonance probe. A series of equally separated 50-ms Gaussian-shaped pulses with a 1-ms delay between the pulses was used for selective saturation of the target protein (GPVI-Fc2), and the total saturation time was 1.5 s. The additional relaxation delay was set to 1.5 s prior to the saturation scheme. The irradiation power of the selective pulses was set to 100 Hz, and the on-resonance irradiation was performed at a chemical shift of −0.5 ppm, while the off-resonance irradiation was applied at −30 ppm.

For TRNOE experiments 445 μg of lyophilized pep-10L peptide was dissolved in 300 μl of 5% D_{2}O/50 mM NaCl/20 mM phosphate buffer (pH 6.5) containing 895 μg of GPVI-Fc2. The final concentrations of the peptide and GPVI-Fc2 were 1 mM and 25 μM, respectively. Two-dimensional TRNOE experiments with a mixing time of 50 ms were performed at 310 K on a Bruker Avance 800-MHz spectrometer with a cryo-cooled triple-resonance probe. For the attenuation of broad resonances of the large protein, the relaxation filter that utilizes spin-locking of z-magnetizations after the preparation delay was applied (25). In this condition, NOE cross-peaks originating from the peptide in the absence of the target protein are mostly suppressed. To minimize intense solvent resonances, the WATERSPECTRE scheme was employed (26).

**Structure Calculations**—Distance restraints for pep-10L upon binding to GPVI-Fc2 were derived from the NOE cross-

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peaks in the TRNOE experiment described above that were converted into distances by volume integration using Sparky4 and the CALIBA program within the CYANA 2.1 software suite (27, 28). An r⁻⁴ distance dependence of the NOE cross-peak intensity is assumed to minimize the potential spin diffusion effect of the NOE distance restraints (29, 30). The NOE intensity between Trp6 Hα-H9 aromatic protons, which corresponds to a distance of 2.6 Å, was used for calibration. A total of 100 structures was calculated by using the torsion angle dynamics protocol. The obtained structures were sorted according to the final value of the target function: the best 20 structures were analyzed with regard to distance and angle violations. Of these 20 structures, the best 10 structures were visualized using MOLMOL.

Binding Site and Binding Pose Prediction—The binding site predictions of GPVI were performed using the following four computer programs, which detect possible ligand-binding sites based on the three-dimensional atomic coordinates of the receptor: “SiteMap” in Schrödinger Suite (Schrödinger, Portland, OR), “Site Detection” in the program “fred_receptor” (OpenEye Scientific Software, Santa Fe, NM), “Site Finder” in the program package MOE (Chemical Computing Group, Montreal, Canada), and “Site ID” in the program SYBYL (Tripos, St. Louis, MO). The crystal structure of GPVI D1-D2 (PDB 2GI7) (14) was used as the input coordinates for prediction. The binding pose prediction of the pep-10L peptide was performed using the PatchDock server (31). The crystal structure of GPVI D1 (PDB 2GI7) (14) and the conformation of the pep-10L determined from the TRNOE data were used for the prediction.

RESULTS

Selection of Peptide-displayed Phages by GPVI-Fc2—To obtain a large amount of peptide ligands with diversity for GPVI, a phage library that displayed random foreign peptides on the N terminus of the major coat protein (gVIII protein) of bacteriophage M13 was constructed. As a target for the panning of the phage display library, recombinant human GPVI-Fc2 expressed by CHO cells was used. After 5 rounds of panning, 10 individual clones were obtained (Table 1). These 10 clones were examined for their GPVI-binding ability in phage enzyme-linked immunosorbent assay (Fig. 1). A recombinant protein irrelevant to GPVI, which has human IgG-Fc, was used as a control protein. Nine clones (Nos. 1, 2, 4, 5, 6, 7, 8, 9, and 10) bound to GPVI-Fc2 and did not bind to the control protein.

Peptides also showed a dose-dependent inhibitory effect (data not shown). These results suggest that pep-5, pep-7, and pep-10 can share the GPO recognition site in GPVI. The sequences of these peptides share more than two acidic residues and four continuous hydrophobic residues (pep-5: Trp⁸-Leu¹⁰-Phe¹⁰-

![FIGURE 1. Binding activity of isolated phage clones to GPVI-Fc2. GPVI-Fc2 (0.1 μg) and control protein (0.1 μg) were coated on a 96-well plate. Individual phage clones were added to each well and incubated at room temperature for 1 h. Detection of the bound phage clones was carried out with the anti-M13-HRP antibody.]

Furthermore, the No. 4, 5, 6, 7, 9, and 10 clones showed less nonspecific binding than that of the No. 1, 2, and 8 clones. These results indicate that the No. 4, 5, 6, 7, 9, and 10 clones have specific binding activity for GPVI.

Inhibitory Effect of Peptides on GPVI-Fc2 Binding to CRP—Six synthetic peptides, pep-4, pep-5, pep-6, pep-7, pep-9, and pep-10, were prepared corresponding to each peptide sequence of Nos. 4, 5, 6, 7, 9, and 10 phage clones. Because the peptide forms the C-terminal homoserinelactone peptide after being released from the modified gVIII protein using CNBr, the N- and C-terminal Met residues were replaced with Ser for the synthetic peptide sequence. The synthetic peptides were tested for their inhibitory effect on GPVI-Fc2 binding to CRP in SPR experiments. CRP, which contains 10 GPO repeats, is a specific agonist for GPVI and is commonly used to study GPVI function (11). The dissociation constant (Kd) of GPVI-Fc2 for CRP is 5.0 × 10⁻⁶ M, as shown in a previous report (18). Three peptides, pep-5, pep-7, and pep-10 (200 μM), showed a >20% inhibitory effect on GPVI-Fc2 binding to CRP (Fig. 2); these peptides also showed a dose-dependent inhibitory effect (data not shown). These results suggest that pep-5, pep-7, and pep-10 can share the GPO recognition site in GPVI.
Pro11; pep-7: Trp8-Trp9-Leu10-Phe11; and pep-10: Trp6-Met7-Tyr8-Phe9). The similarity between the peptides suggests that acidic residues and/or hydrophobic residues may be involved in GPVI binding.

The pep-10 peptide had the strongest inhibitory effect on GPVI binding to CRP (Fig. 2). Therefore, this peptide was the focus of further experiments. Another synthetic peptide, pep-10L, which replaces the CNBr-cleavable Met residue in the pep-10 sequence with a Leu residue, was prepared. The pep-10L peptide had an inhibitory effect on GPVI-Fc2 binding to CRP equal to pep-10 (Fig. 2). This result indicates that the inhibitory effect of pep-10L is comparable to that of pep-10 and that the Met residue is replaceable with Leu without a loss of activity. The pep-10L peptide dose-dependently inhibited GPVI-Fc2 binding to CRP (supplemental Fig. S1), and its \( K_i \) was calculated to be 180 \( \mu \)M (Table 2). This result suggests that the pep-10L peptide is a full antagonist for GPVI-Fc2 binding to CRP.

**Inhibitory Effect of pep-10L on GPVI-Fc2 Direct Binding to the GPO Sequence**—Next, the effect of the pep-10L peptide on GPVI binding to human type I collagen and the GPO-1 peptide was investigated. Type I collagen is a native ligand for GPVI and is involved in platelet adhesion and activation in the vascular subendothelium (32). Unlike in CRP, there are very few contiguous runs of GPO triplets in type I collagen. Type I collagen has five GPOGPO motifs and over 30 single GPO triplets. GPOGPO or GPO have been identified as the minimum recognition motifs for GPVI, and the GPO recognition site on the GPVI surface is a primary collagen-binding site (12). Furthermore, a GPVI antagonist that is able to inhibit platelet aggregation implies the need for inhibiting GPVI binding to the minimum recognition motif in collagen (12). Therefore, to examine the inhibitory effect of the pep-10L peptide on the direct interaction of GPVI with its minimal binding motif, the GPO-1 peptide was prepared based on the “cystine-knot” disulfide link (17). The GPO-1 peptide has the homogeneous structure of a single GPO-containing triple-helical homotrimer peptide (data not shown). The \( K_i \) values of GPVI-Fc2 for immobilized human type I collagen and GPO-1 were 1.1 \( \times 10^{-5} \) M and 2.4 \( \times 10^{-5} \) M, respectively. Pep-10L inhibited GPVI-Fc2 binding to both collagen and GPO-1 in a dose-dependent manner (supplemental Fig. S2), and their \( K_i \) values are shown in Table 2. These results suggest that GPVI-Fc2 recognizes GPO in type I collagen and that the pep-10L peptide can share the GPO recognition site(s) on GPVI.

**Ala Scanning of pep-10L**—First, the amino acid side-chain contribution to GPVI-Fc2 binding of pep-10L was evaluated by Ala replacement of each residue of the pep-10L peptide library sequence (positions 2–11). Ten Ala-substituted pep-10L analogs were prepared and tested for their inhibitory effect on GPVI-Fc2 binding to CRP (Fig. 3). The substitution of Trp6 and Phe9 with Ala generated an inactive analog (pep-10L-5 and pep-10L-8). Ala replacement of Leu7 and Tyr8 led to a slight reduction in the inhibitory effect on GPVI-Fc2 binding to CRP (pep-10L-6 and pep-10L-7). Interestingly, the Ala replacement of Asp5 and Ser10 produced increased inhibitory activity (pep-10L-4 and pep-10L-9). Ala substitutions of Ser2, Asp3, Thr4, and Thr11 did not affect inhibition (pep-10L-1, pep-10L-2, pep-10L-3, and pep-10L-10). These results suggest that the 6-amino acid sequence Asp5-Trp6-Leu7-Tyr8-Phe9-Ser10 is important for the GPVI-Fc2-binding activity of pep-10L.

**Identification of Residues in the pep-10L Peptide Interacting with GPVI-Fc2**—Next, an STD NMR experiment was performed to identify the residues in the pep-10L peptide that interact with GPVI-Fc2. Radio frequency irradiation, which is selectively applied to GPVI-Fc2 protons, causes proton resonance saturation of GPVI-Fc2; this saturation is transferred to the bound peptide through the binding interface. Peak intensities of contact residues are more affected than those of noncontact residues. The intensity ratio (with/without irradiation at \( -0.5 \) ppm) of each proton in the pep-10L peptide is shown in supplemental Fig. S3. A decreased intensity ratio \((<0.8)\) was observed in the protons of Trp9, Leu7, Phe9, and Ser10, indicating that these residues are in contact with GPVI-Fc2. Conformation of the pep-10L Peptide Bound to GPVI-Fc2—The three-dimensional structure of the pep-10L peptide bound to GPVI-Fc2 was determined using TRNOE analysis. In the NOESY spectrum of the pep-10L peptide in the presence of GPVI-Fc2, a large number of intense negative NOE cross-peaks were observed, whereas only a few NOE cross-peaks were observed in the NOESY spectrum of the peptide alone (data not shown). This indicates that these NOE cross-peaks reflect the conformation of the pep-10L peptide bound to GPVI. In the spectrum, several TRNOE cross-peaks were observed between sequential amide protons from Asp3 to Ser10 (data not shown), which indicates that the peptide partially adopts a helical conformation in the bound state. Structure calculations of pep-10L bound to GPVI-Fc2 were performed with CYANA 2.1 using 93 distance constraints (55 intrasresidue and 38 interresidue: 26...
The GPVI-bound conformation of pep-10L is shown in Fig. 4A. 20 structures were chosen of the 100 generated, and no structure had NOE violations > 0.2 Å. The root mean square deviation to a mean structure calculated for the backbone atoms and the heavy atoms of residues 4–10, which corresponds to the central part of the peptide library portion, are 0.36 ± 0.21 Å and 0.85 ± 0.20 Å, respectively. The Ramachandran appearance in the most favored regions, additional allowed regions, generously allowed regions, and disallowed regions for residues 4–10 are 52.9, 45.7, 1.4, and 0.0%, respectively. N- (residues 1–3) and C-terminal (residues 11 and 12) regions for residues 4–10 are 52.9, 45.7, 1.4, and 0.0%, respectively. The figures were generated using the program MOLMOL (24).

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Sequential (|i − j| = 1) and 12 medium range (1 < |i − j| ≤ 4) derived from the TRNOE spectrum.

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Interestingly, Ala replacements of Asp5 and Ser10 produced increased inhibition. Substituting Asp5 or Ser10 for Ala conceivably increases the hydrophobic interaction with GPVI. These findings are useful in designing new peptides or peptide-mimetics that have higher binding activity than pep-10L.

DISCUSSION

To experimentally obtain structural and interaction information about a peptide ligand in the GPVI-bound state, peptides that bound to GPVI in a phage display library were screened. Through SPR experiments using synthetic peptides, the pep-10L peptide was found to have an inhibitory effect on GPVI binding to CRP, collagen, and the GPO-1 peptide. The pep-10L peptide showed near-complete inhibition of GPVI binding to CRP, type I collagen, and the GPO-1 peptide, and supposedly shares the GPO recognition site(s) on the GPVI surface. A GPVI antagonist that is able to inhibit platelet aggregation needs to inhibit GPVI binding to the minimum recognition motif in collagen (12). Therefore, the pep-10L peptide has the potential to serve as a GPVI antagonist, exerting an anti-thrombogenic effect.

Ala scanning of pep-10L as shown in SPR competitive experiments demonstrated that the Asp5–Trp6–Leu7–Tyr8–Phe9–Ser10 sequence is important for binding to the GPO recognition site on the GPVI surface. To discriminate between directly interacting residues and nonbinding residues in the pep-10L peptide, an STD NMR experiment was performed. In Fig. 4B, the result from the STD experiment is mapped onto the surface representation of the peptide. This experiment revealed that Trp6, Leu7, Phe9, and Ser10 residues in the pep-10L peptide interacted with GPVI. Through TRNOE experiments, we have shown that the central part of pep-10L (Asp5–Phe9) in the GPVI-bound state adopts a helical conformation. The obtained structure reveals that the side chains of Trp6, Leu7, and Phe9 form a hydrophobic side in the helix, while the side chain of Tyr8 faces the opposite direction. As shown in the Ala scanning experiments and in the STD NMR experiments, this hydrophobic portion may be important for the inhibitory activity of the pep-10L peptide. The Asp5 and Tyr8 residues seem to be required to support the structure of the hydrophobic portion. Interestingly, Ala replacements of Asp5 and Ser10 produced increased inhibition. Substituting Asp5 or Ser10 for Ala conceivably increases the hydrophobic interaction with GPVI. These findings are useful in designing new peptides or peptide-mimetics that have higher binding activity than pep-10L.

Recently, the importance of the direct binding of a small molecule to the “hot spot” of a protein-protein interface, in order for the former to inhibit protein-protein interactions, has been reported (33). This report suggests the presence of a hot spot, which is a hydrophobic pocket, as the binding site of the pep-10L peptide on the GPVI surface. GPVI is a member of the immunoglobulin superfamily and has two immunoglobulin-like extracellular domains (D1 and D2). On the crystal structure of GPVI (D1–D2 domain), there are several hydrophobic sur-
faces formed by hydrophobic residues (Ala, Val, Leu, Ile, Met, Phe, Trp, Tyr, and Pro; yellow surfaces in Fig. 5). Predictions of the peptide-binding site were performed using the human GPVI D1–D2 x-ray coordinate (PDB 2GI7) (14) and four computer programs (SiteMap, Site Detection, Site Finder, and Site ID). The first three predicted the hydrophobic groove formed by Leu\(^{39}\), Leu\(^{53}\), Phe\(^{54}\), Pro\(^{56}\), Leu\(^{62}\), and Tyr\(^{66}\) in the D1 domain (Fig. 5, yellow region indicated by arrow) as the first candidate for a spatial peptide-binding site on GPVI. Site ID predicted the groove as the second candidate. Further, docking calculations between the structure of the pep-10L peptide, which was determined by the TRNOE experiment, and the crystal structure of GPVI D1 (PDB 2GI7) (14), were performed using the PatchDock server. Because the N- and C-terminal regions of the peptide are not well defined in the NMR structure, Thr\(^4\)–Ser\(^{10}\) was used for the docking. All of the top 10 binding poses showed that the pep-10L peptide bound to the hydrophobic groove. The best scored pose is shown in Fig. 6. As illustrated in Fig. 6B, the hydrophobic side in the helix of pep-10L sticks in the hydrophobic groove of GPVI D1. This hydrophobic interaction is in good agreement with the results of Ala scanning and the STD NMR experiment. Furthermore, the hydrophobic groove on GPVI (Fig. 6B, yellow surface) is consistent with the hydrophobic portion of the putative CRP binding groove (14). Taken together, the hydrophobic groove on GPVI (Fig. 6B, yellow surface) is reasonable as a candidate for the pep-10L binding site. NMR analyses are currently being performed to experimentally identify the binding site(s) of pep-10L on the GPVI surface.

We performed structural and interaction analysis of the pep-10L peptide, which is a novel GPVI-binding peptide ligand selected from a phage display library. Our results suggest that the pep-10L peptide shares the GPO recognition site on GPVI, although the obtained peptide seems to have little structural similarity to the triple helical GPO motif. We have discovered the GPVI-bound conformation of the pep-10L peptide and identified the critical residues for interaction with GPVI. Although the obtained peptide might be a low potency molecule at present, it is well known that the stabilization of the active conformation of a ligand enhances its activity by up to three orders of magnitude (34–36). Because the active conformation of the peptide has been determined in this study, the obtained structural information would be useful for improving the affinity of the peptide and/or the design of small molecules that bind to GPVI with high potency. Further, the pep-10L peptide could be used as a probe for drug screening; i.e., screening for compounds that inhibit the peptide probe and GPVI interaction could be performed. This holds promise for obtaining compounds that have a higher affinity for GPVI than the pep-10L peptide.

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