Collagen-related peptide is a selective agonist for the platelet collagen receptor Glycoprotein VI. The triple helical peptide contains ten GPO triplets/strand (single letter amino acid nomenclature, where O is hydroxyproline) and so over-represents GPO compared with native collagen sequence. To investigate the ability of Glycoprotein VI to recognize GPO triplets in a setting more representative of the collagen sequence, we synthesized a set of triple helical peptides containing fewer GPO triplets, varying their number and spacing within an inert (GPP)ₙ backbone. The adhesion of recombinant human Glycoprotein VI ectodomain, like that of human platelets, to these peptides increased with their GPO content, and platelet adhesion was abolished by the specific anti-Glycoprotein VI-blocking antibody, 10B12. Platelet aggregation and protein tyrosine phosphorylation were induced only by cross-linked peptides and only those that contained two or more GPO triplets. Such peptides were less potent than cross-linked collagen-related peptide. Our data suggest that both the sequences GPOGPO and GPO⋯⋯⋯⋯GPO represent functional Glycoprotein VI recognition motifs within collagen. Furthermore, we propose that the (GPO)₄ motif can support simultaneous binding of two glycoprotein VI molecules, in either a parallel or anti-parallel stacking arrangement, which could play an important role in activation of signaling.

Damage to the blood vessel endothelium results in the exposure of underlying extracellular matrix proteins, including the fibrillar collagens I and III, both abundant in that location. Interaction of circulating platelets with collagen is a multistage process involving several receptors in which the activatory collagen receptor glycoprotein VI (GpVI) plays a central role in activation of signaling. The adhesion of recombinant human Glycoprotein VI ectodomain, like that of human platelets, to these peptides increased with their GPO content, and platelet adhesion was abolished by the specific anti-Glycoprotein VI-blocking antibody, 10B12. Platelet aggregation and protein tyrosine phosphorylation were induced only by cross-linked peptides and only those that contained two or more GPO triplets. Such peptides were less potent than cross-linked collagen-related peptide. Our data suggest that both the sequences GPOGPO and GPO⋯⋯⋯⋯GPO represent functional Glycoprotein VI recognition motifs within collagen. Furthermore, we propose that the (GPO)₄ motif can support simultaneous binding of two glycoprotein VI molecules, in either a parallel or anti-parallel stacking arrangement, which could play an important role in activation of signaling.

Each fibrillar collagen strand contains ~1000 amino acid residues arranged in the triple helical COL domain, comprised of (G-X-X′) repeats, where X is often proline and X′, hydroxyproline (O). Glycine must occur at every third residue to allow the structure to fold as a triple helix (5). Collagen-related peptide (CRP) (GCO-(GPO)₁₀-GCOG) readily adopts this triple helical structure (6) but is a highly potent platelet agonist only after cross-linking (CRP-XL) that introduces quaternary structure (7–9). CRP-XL has been shown to interact with GpVI (10, 11) and has been widely adopted as a specific tool with which to investigate the platelet-collagen interaction. In contrast, soluble monomeric CRP is at best a weak agonist (12).

GPO triplets are abundant in collagens, forming ~10% of the fibrillar collagen COL domain, although very few contiguous runs of GPO triplets occur; exceptions are the N-terminal domain of collagens I (α1) and III (α1), where four and three GPO triplets are present, respectively, and the C-terminal domain of collagen I α1, where five such triplets occur. The present work was designed to help understand how the organization of GPO triplets in the native collagens can activate GpVI despite the relative rarity of GPO-rich sequence.

Horii et al. (13) recently described the crystal structure of the immunoglobulin (Ig)-like ligand-binding domain of GpVI and proposed a model for its interaction with collagen after docking with CRP-like structures. This study confirmed the importance of the apical residues in domain 1 of GpVI previously identified by mutagenesis (14, 15) and presented a complex in which the long axis of GpVI domain 1 is roughly perpendicular to that of the collagen helix. The authors also show by analytical ultracentrifugation that monomeric CRP can interact with more than one copy of GpVI (13). This proposed binding mechanism is very different from that recently described for the complex of collagen with the Ig-fold-containing Staphylococcus aureus CNA protein (16). CNA wraps the triple helix between its tandem Ig domains with a long (9-residue), flexible interdomain linker. The major binding surface on domain N2 of CNA is predominantly hydrophobic, as proposed similarly for the GpVI-CRP interaction (13). The periphery of the proposed binding groove on GpVI contains polar and positively charged residues that may be responsible for the specificity of GpVI for GPO rather than GPP peptides.

The platelet reactivity of CRP has been shown to reside in the hydroxyproline content of its GPO triplets (17), but little is known of how they must be distributed within the collagen.
primary sequence or within the three-dimensional organization of the collagen fiber to induce platelet activation. GPO may also be crucial for other interactions such as the recognition of keratinocyte growth factor by collagen (18) or the regulation of the immune receptor, LAIR-1 (19), but again, there is little understanding of the determinants of the activation process at the level of collagen structure.

Collagen-like peptides whose triple helical structure is stabilized by terminal GPP repeat modules have been used previously to identify the minimal integrin α2β1 collagen binding motifs (20). Here, we have investigated the adhesion of platelets and of the GpVI ectodomain to a series of collagen-like model peptides containing GPO motifs of increasing length within (GPP)n sequences, with overall peptide length conserved. We also investigated the ability of these peptides to activate platelets via GpVI, measured as platelet aggregation and protein tyrosine phosphorylation. We show that the smallest effective GpVI recognition motifs contain two GPO triplets, either adjacent or separated by four GPP triplets. This evidence is compatible with a model for multivalent interactions on the platelet surface in which individual GpVI molecules bind to separate GPO triplets within a single triple helical peptide, while two adjacent GPO motifs provide a stronger binding site for a single GpVI molecule. Extended GPO motifs may also induce dimerization of GpVI in side-by-side stacks. These hypotheses were tested by docking CRP-like molecules onto the GpVI structure. Our data also underline the requirement for clustering of GpVI on the platelet surface following interaction with GPO polymers before activatory signals ensue.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human platelets were isolated from fresh whole blood for adhesion and aggregation assays and from pooled platelet concentrate for tyrosine phosphorylation assays. Both blood and concentrate were obtained from consenting donors of the National Blood Service Cambridge, Long Road, Cambridge, UK, in accordance with the Helsinki protocol. Monomeric type I collagen for use in solid phase adhesion assays was purified from bovine skin, following limited pepsin digestion, as described previously (21). Collagen fibers were a gift from Ethicon Corp., Somerville, NJ. Anti-(human integrin α2-subunit) monoclonal antibody 6F1 (23) was a generous gift from Dr. B.S. Coler (Mount Sinai Hospital, New York, NY). Calmodulin-tagged, recombinant human GpVI Ig-like domains (hD1D2) and the human GpVI-blocking recombinant antibody 10B12 were produced as described elsewhere (14). Monoclonal mouse anti-phosphotyrosine antibody 4G10 was from TCS Biologicals, Buckingham, UK. Horseradish peroxidase-linked sheep anti-mouse IgG (NA931) was from Amersham Biosciences. Chemicals were from Sigma-Aldrich unless otherwise stated. The RGD mimetic, GR144053F, was from Calbiochem. Hybond C nitrocellulose (RPN303C) and rainbow molecular weight markers (RPN756) were from Amersham Biosciences.

**Peptide Synthesis and Purification**—Peptides were synthesized by Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry as C-terminal amides on Tentagel R RAM resin and purified as described previously (24).

**Peptide Melting Temperature Determination**—Peptides were dissolved at 5 mg/ml in 0.01 M acetic acid, incubated at 60 °C for 25 min, and then placed at 4 °C overnight to encourage optimal triple helix formation. Stability of triple helical peptides was assessed the following day by polarimetry in a Jasco P-1020 polarimeter equipped with a sodium lamp (589 nm). The peptide sample was de-gassed under vacuum for 1–2 min, 700 μl was placed in a 5-cm path length, water-jacketed cell (connected to a Lauda RE104 thermostatted waterbath fitted with a circulation pump), and the optical rotation was measured at 5 °C intervals between 5–95 °C. 15 min was allowed for thermal equilibration at each temperature before taking three optical rotation readings, each averaging over 20 s. Peptide melting temperatures were calculated by fitting the melting curve to a theoretical melting equation by non-linear regression.

**Peptide Cross-linking**—Peptides were cross-linked using a modification of a previous method (9). Peptides were dissolved in water, adjusted to 0.1 M NaHCO₃, and then 1.5 molar equivalents of the heterobifunctional cross-linking agent SPDP (N-succinimidyl 3-(2-pyridyldithio)propionate) in dry ethanol was added. The vials were flushed with nitrogen and rotated at room temperature for 1 h. The cross-linked peptides were transferred to QuixSep™ Micro Dialyzers from Perbio Science Ltd., Cheshire, UK, assembled using CelluSep H1 dialysis membrane (nominal molecular mass cut-off 1 kDa), and dialyzed against 1 liter of 0.01 M acetic acid at 4 °C three times, each for 2 h, with constant stirring.

**Static Platelet Adhesion and Ligand Binding Assays for Recombinant GpVI**—Platelet adhesion to ligands adsorbed to Immulon 2 96-well plates was determined colorimetrically (25). The ligand binding assay for measurement of the binding of hD1D2 to triple helical peptides was performed as previously described (15).

**Platelet Aggregation**—Platelet-rich plasma was isolated from fresh whole blood by centrifugation at 200 × g for 12 min. After removal of platelet-rich plasma, the blood sample was spun for 10 min at 1200 × g, yielding platelet-poor plasma, which was used to calibrate the aggregometer. Platelet aggregation in platelet-rich plasma was measured turbidimetrically at 37 °C using a constant stirrer speed of 1100 rpm in a Chronolog 490-4D aggregometer (Labmedics Ltd., Stockport, Cheshire, UK).

**Platelet Protein Tyrosine Phosphorylation Assay**—Platelet concentrate (10 ml) was spun at 200 × g for 15 min to remove red blood cells. The platelet-rich supernatant was transferred to a new tube, and apyrase was added (0.25 units/ml final concentration) to scavenge ADP and then spun at 600 × g for 15 min. The supernatant was removed, and the platelet pellet was resuspended in 4 ml of loading buffer (145 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM MgSO₄, 0.5 mM EGTA, 10 mM N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid (HEPES, pH 7.36) and spun at 600 × g for 10 min. Platelets were resuspended to ~1 × 10⁸ cells/ml. Aliquots (45 μl) of platelet suspension were added to 5 μl of agonist, mixed briefly, and incubated at 37 °C for 3 min. The reaction was terminated by the addition of an equal volume of 2× Laemml buffer (130 mM Tris, 69.3 mM SDS, 0.373 mM bromphenol blue, 20% v/v glycerol, 10% v/v 2-mercaptoethanol, pH 6.8) and the mixture heated at 100 °C.
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**TABLE 1**

Amino acid sequence of model triple helical peptides used in this study. The table gives the peptide sequence using single letter nomenclature (O = hydroxyproline). Melting temperatures ($T_m$) were determined in triplicate by polarimetry.

| Peptide | Sequence | $T_m \pm$ S.D. ($^\circ$C) |
|---------|----------|--------------------------|
| CRP     | GCO-(GPO)$_{10}$-GCOG | 82.3 ± 1.4 |
| GPP10   | GCP-(GPO)$_{10}$-GCPG | 45.8 ± 0.8 |
| GPO1    | GCP-(GPP)$_{5}$-GPP-GCPG | 45.6 ± 0.7 |
| GPO2    | GCP-(GPP)$_{5}$-(GPO)$_{10}$-GPP-GCPG | 49.7 ± 0.5 |
| GPO4    | GCP-(GPP)$_{5}$-(GPO)$_{5}$-GPP-GCPG | 55.6 ± 0.4 |
| GPO6    | GCP-(GPP)$_{5}$-(GPO)$_{5}$-GPP-GCPG | 59.9 ± 1.8 |
| GPP4GPO2| GCP-(GPP)$_{5}$-(GPO)$_{5}$-GPP-GCPG | 50.3 ± 0.4 |
| GPP2GPO4| GCP-(GPP)$_{5}$-(GPO)$_{5}$-GPP-GCPG | 52.7 ± 1.5 |

for 5 min. Platelet protein samples were separated by SDS-PAGE, transferred to nitrocellulose, and probed with mouse monoclonal anti-phosphotyrosine antibody (4G10) at a 1:2500 dilution in blocking buffer (20.0 mM Tris, 136.8 mM NaCl, 0.1% v/v Tween 20, 5% w/v bovine serum albumin, 0.02% sodium azide, pH 7.6) on a rocker at room temperature for 1 h. Blots were then washed twice for 5 min and twice for 20 min in TBST (20.0 mM Tris, 136.8 mM NaCl, 0.1% v/v Tween 20, pH 7.6) and incubated in sheep anti-mouse IgG horseradish peroxidase-linked secondary antibody (NA931) at a 1:10,000 dilution in TBST containing 1% w/v bovine serum albumin on a rocker for 1 h at room temperature. The blots were washed further in TBST, twice for 5 min and twice for 20 min. Tyrosine-phosphorylated protein bands were detected by enhanced chemiluminescence.

**RESULTS**

Two GPO Triplets in a Triple Helical Peptide Support GpVI-mediated Platelet Adhesion—To identify a minimal GpVI binding motif, a set of triple helical peptides was synthesized containing different numbers and spacings of GPO triplets within the parent GPP polymer. Each peptide was 37 amino acid residues in length, and cysteine residues were incorporated near the N and C termini to allow them to be cross-linked into a polymeric structure (9). All these peptides had a melting temperature above 45°C (Table 1), which confirms their triple helical state under the assay conditions used here. In general, peptide stability was proportional to the number of GPO triplets present, in agreement with previous work (28).

Platelets adhered only slightly and non-significantly to the parent peptide, GPP10, as shown previously (17). Significant adhesion to Peptide GPO1 was observed (Fig. 1). However, Peptide GPO2 supported greater platelet adhesion, of ~50% relative to CRP. Platelet adhesion to Peptide GPO2 was greater than to GPO1 but less than to Peptide GPO4 (each p < 0.01, repeated measures analysis of variance). Platelet adhesion to CRP could not be distinguished from that to Peptides GPO4 and GPO6. The GpVI-blocking antibody 10B12, used at 10 µg/ml, abolished platelet adhesion to each of the GPO-containing peptides (Fig. 2). Adhesion to the peptide that contained the integrin binding motif GFOGER (19) was unaffected by 10B12, whereas that to both monomeric and fibrous collagen was slightly but significantly reduced (p < 0.02, paired sample t-tests).
Binding of Recombinant GpVI to GPO-containing Peptides—No greater binding of hD1D2 occurred to wells coated with Peptide GPP10 than was observed to the blocked plate, in line with previous findings (14). Peptide GPP10 was therefore used to determine nonspecific binding activity. Specific binding of hD1D2 to Peptide GPO1 was slight but approximated to a linear dependence on D1D2 concentration at up to 300 μg/ml ($r^2 = 0.86$) with slope differing significantly from zero ($p < 0.0001$) (Fig. 3). Binding to Peptide GPO2 was clearly detectable. We observed a stepwise increase in binding of hD1D2 as the GPO content of the peptide substrates increased, so that Peptide GPO4 supported around twice as much hD1D2 binding as Peptide GPO2 at all concentrations tested, with a similar increment with CRP as substrate. Peptide GPO6 supported intermediate levels of adhesion (Fig. 3). These data are qualitatively similar to those obtained from platelet binding experiments (Figs. 1 and 2).

Cross-linked Triple Helical Peptides Containing Two or More GPO Triplets Induce Tyrosine Phosphorylation of Platelet Proteins—Platelet protein tyrosine phosphorylation provides a sensitive index of platelet activation. Stimulation of platelets with monomeric (non-cross-linked) triple helical GPO-containing peptides did not result in large increases in tyrosine phosphorylation over controls (Fig. 4). CRP was the only monomeric peptide that increased protein tyrosine phosphorylation visibly, and then only one protein band, ~70 kDa, responded, requiring a dose of ~50 μg/ml. To mimic the polymeric quaternary structure of collagen, triple helical peptides can be cross-linked between cysteine side chains and primary amino groups with the heterobifunctional cross-linking reagent SPDP, (N-succinimidyl 3-(2-pyridyldithio)propionate) (9). Throughout the description that follows, cross-linked forms of peptides are denoted “peptide-XL”. In contrast to monomeric CRP, CRP-XL at 5 μg/ml induced maximal platelet activation in line with previous reports (7, 29). The CRP-XL-stimulated lane of Fig. 4D is overexposed to reveal the minor activity of monomeric CRP in the adjacent lanes. Stimulation with monomeric CRP at 500 μg/ml caused less protein tyrosine phosphorylation than when used at 50 μg/ml ($n = 2$).

Peptides GPP10-XL and GPO1-XL induced no consistent increase in tyrosine phosphorylation, but Peptide GPO2-XL caused substantial tyrosine phosphorylation, obvious at 100 μg/ml of peptide, but lower than maximal activity (CRP-XL at 5 μg/ml) even when peptide was used at 500 μg/ml (Fig. 5B). Peptides GPO4-XL and GPO6-XL were more potent, inducing higher levels of tyrosine phosphorylation at 20 and 10 μg/ml, respectively (Fig. 5, C and D). Peptides GPP4GPO2-XL and GPP2GPO4-XL, like GPO2-XL, were less potent ligands than Peptides GPO4-XL and GPO6-XL (Fig. 5, E and F).

**FIGURE 3.** Binding of recombinant GpVI (hD1D2) to GPO peptides measured by enzyme-linked immunosorbent assay. hD1D2-calmodulin was allowed to bind to immobilized ligands for 2 h at room temperature and subsequently detected using a peroxidase-conjugated CaM binding peptide. Data are mean ± S.E. of absorbances from four independent experiments, each performed in triplicate, after subtraction of background binding to GPP10 in each experiment.

**FIGURE 4.** Monomeric GPO-containing triple helical peptides do not induce platelet protein tyrosine phosphorylation. Platelets were stimulated for 3 min at 37 °C with monomeric Peptides GPP10 and GPO1 (A), GPO2 and GPO4 (B), GPO6 and GPP4GPO2 (C), GPP2GPO4 and CRP (D). Platelets were stimulated and lysed, and proteins were separated on 8% SDS-PAGE and transferred to nitrocellulose. Phosphotyrosine was detected with monoclonal anti-phosphotyrosine antibody (4G10) as described under “Experimental Procedures.” The blots shown are representative of three experiments. Numbers above lanes denote peptide concentration (μg/ml), 0 denotes platelets stimulated with vehicle alone (0.01 M acetic acid, 10% v/v), and C denotes CRP-XL (5 μg/ml). Molecular mass markers are shown on the right.
Stimulation of platelets with cross-linked GPO-containing peptides led to phosphorylation of the same protein bands as with CRP-XL, albeit of lower intensity.

**Platelet Aggregation**—Monomeric forms of GPO-containing triple helical peptides did not induce aggregation even when tested at 2 mg/ml (data not shown). Peptides GPP10-XL and GPO1-XL were inactive at up to \( \sim 150 \) and 300 \( \mu \)g/ml, respectively (Fig. 6A), in agreement with the tyrosine phosphorylation data (Fig. 5). The response to Peptide GPO2-XL varied between donors: in five of eleven experiments using different donor platelets, Peptide GPO2-XL did not cause any aggregation at up to \( \sim 1 \) mg/ml. However, in the remaining six experiments, Peptide GPO2-XL induced full aggregation at 5–142 \( \mu \)g/ml. On those occasions when GPO2-XL successfully induced full platelet aggregation, there was a substantial delay of \( \sim 100 \) s between addition of agonist and onset of full aggregation (Fig. 6A, iii), compared with a delay of \( \sim 20 \) s with CRP-XL.

The minimum dose of Peptide GPP4GPO2-XL that caused full aggregation was comparable with that required for GPO2-XL (Fig. 6B, i). In two of eight trials, GPP4GPO2-XL at up to 650 \( \mu \)g/ml did not cause platelet aggregation, but in the remaining experiments aggregation was achieved with 20 \( \mu \)g/ml and above. Peptide GPP2GPO4-XL induced full aggregation in all six donors tested at doses of 2 \( \mu \)g/ml and above. Peptide GPO4-XL induced platelet aggregation at 1 \( \mu \)g/ml and above, and GPO6-XL induced aggregation at 0.5 \( \mu \)g/ml and above (Fig. 6B, iii and iv). The potency of cross-linked GPO-containing triple helical peptides for induction of platelet aggregation is summarized, relative to the activity of CRP-XL, in Table 2.

**DISCUSSION**

The aim of this study was to determine the minimum arrangement of GPO triplets within CRP-like peptides that are recognized by GpVI and so activate platelets. To this end, we synthesized a set of triple helical peptides containing different numbers and spacing of GPO triplets within a host sequence of \((GPP)\)\(_n\). The complete abolition by antibody 10B12 of platelet adhesion to the GPO-containing Peptides GPO2, GPO4, and CRP showed the interaction to be mediated by GpVI.

The weak, but significant, binding of platelets and D1D2 to Peptide GPO1 demonstrates the ability of a single GPO triplet to interact with GpVI. However, when a single additional adja-
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cent GPO triplet was introduced, as in Peptide GPO2, a marked increase in binding of both platelets and D1D2 was observed, suggesting that this peptide provides a more complete recognition motif for each individual GpVI molecule.

For both platelets and D1D2, a significant increase in binding was observed between Peptide GPO2 and Peptide GPO4 and a further progressive increase as the GPO peptide content increased to six and ten triplets. The axial dimension of the GPOGPO motif in triple helical peptides is sufficiently small, \(\sim 15\) Å, to exclude the possibility that GPOGPO can bind more than one copy of GpVI.

An important interpretation of our data together with recent structural evidence (13) is that GPO-containing peptides of increasing length have both greater capacity and increased affinity for D1D2, the latter accounted for by cooperativity of binding. These conclusions are limited by the restricted availability of recombinant D1D2 for the saturation binding experiments that would define the capacity of the peptides exactly.

The recent solution of the GpVI structure, and the previous high resolution structures for GPP and GPO polymers (6, 30), has allowed a complex between the two to be proposed (13). Site-directed mutagenesis of GpVI has identified residues that modulate binding of collagen and CRP (14, 15), and it is interesting that the docking of both GPP and GPO polymers with GpVI suggested a binding trench on the surface of GpVI that contains these same crucial residues, Lys-41 and Lys-59. The resulting model had no absolute requirement for hydroxylation of the X prolines, although data shown here confirm that GPP polymers bind little GpVI, as we have found previously (17).

Inspection of models of these peptides docked onto GpVI allows us to propose that the minimum interacting species appears to be a cluster of 3 O residues occupying the same position in adjacent strands of the triple helix, designated O\(\text{A}\), O\(\text{B}\), and O\(\text{C}\) in Fig. 7C and separated only by the single amino acid stagger that occurs between strands. From our data, it is clear that GPOGPO clusters support stronger binding, however. Additional O residues may be involved, O\(\text{A}\), O\(\text{B}\), and O\(\text{C}\), located one triplet toward the N terminus of the triple helix, and these 6 O residues, comprising longitudinal separation of \(\sim 15\) Å, may constitute the crucial footprint of collagen upon the surface of a single GpVI molecule. Flexible side chains on GpVI tend to embrace the peptide and can interact with all three strands. On its own, each such GPO cluster will support only weak GpVI binding.

Longer GPO-containing peptides such as Peptide GPO4 provide further binding energy, as, for example, O\(\text{A}\), O\(\text{B}\), and O\(\text{C}\) may interact with the collagen binding trench of GpVI (Fig. 7C). The binding trench itself appears to be four GPO triplets in length, including the triplet closest to Arg-166 may interact weakly, if at all. I

**TABLE 2**

| Peptide                  | % donor plates fully aggregating | Number of trials | Relative EC\(_{\text{max}}\) |
|--------------------------|---------------------------------|------------------|-----------------------------|
| CRP-XL                   | 100                             | 31               | 1                           |
| GPP10-XL                 | 100                             | 10               | >15000                      |
| GPO1-XL                  | 0                               | 5                | >6000                       |
| GPO2-XL                  | 55                              | 11               | 100-1420                    |
| GPO4-XL                  | 100                             | 15               | 4-1000                      |
| GPO6-XL                  | 100                             | 8                | 2.5-50                      |
| GPP4GPO2-XL              | 75                              | 8                | 40-8500                     |
| GPP4GPO4-XL              | 100                             | 15               | 40-1200                     |

**FIGURE 6. Platelet aggregation evoked by cross-linked GPO peptides.** The ability of each cross-linked GPO-containing peptide to induce platelet aggregation is shown. The indicated level of peptide is either the minimum required to elicit a full aggregatory response or the maximum dose applied where peptides caused no aggregation. Discontinuity in baseline indicate addition of agonist. Traces are single representative examples of 3 determinations in platelet-rich plasma at 37°C.
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Residues in different strands, is sufficiently large as to exclude the possibility that a single copy of GpVI can interact with both GPO triplets. Thus we can conclude that, although more than one copy of the receptor must interact with the separated GPO and GPOGPO motifs in Peptide GPP4GPO2 and Peptide GPP2GPO4, a single copy of GpVI must bind to GPOGPO in Peptide GPO2 and to each GPO or GPOGPO motif in Peptides GPP4GPO2 and GPP2GPO4. The enhanced reactivity of GPP4GPO2 suggests that two linked GpVI receptors at the platelet surface interact with the separate clusters of GPO triplets in each of these peptides.

Some of us used such evidence to predict the occurrence of GpVI as a dimer (31), whereas Miura et al. (32) found dimeric Fc fusion constructs, but not monomeric forms, of GpVI could bind collagen in vitro. The crystal structure of GpVI presented by Horii et al. (13) indeed contained a dimeric structure, maintained by D2-D2 interaction in the absence of CRP, such that the first domain of each points in opposite directions along the same axis, although the occurrence of this GpVI dimer conformation on the platelet surface has yet to be verified. However, although each GPO cluster in Peptides GPP4GPO2 and GPP2GPO4 could interact with a single GpVI protomer within such a preformed GpVI dimer, a single GpVI dimer could not interact simultaneously with both GPO clusters in these peptides. This is because the orientation of the putative binding groove on the surface of D1 is perpendicular to the long axis of the GpVI dimer observed in the crystal. This suggests that another mechanism of association of GpVI, direct or indirect, GpVI would be capable of sufficient interactions with hydroxyprolines within the GPO2 motif to mediate specific binding.

We have recently demonstrated that the function-modifying antibody 1C3, binding to an epitope on one face of hD1D2, can prevent its recognition of extended GPO motifs on a helix by a second D1D2 molecule (35). This inhibition is compatible with the stacking mechanism proposed here.

Implications for Signaling—Although the intracellular cascades activated by GpVI are increasingly well understood (36), the extracellular events that lead to the activation of GpVI are poorly defined. Monomeric CRP induces limited tyrosine phosphorylation, very slight compared with CRP-XL, without going on to support full platelet aggregation. The inability of any monomeric peptide other than CRP to stimulate protein tyrosine phosphorylation indicates that simple occupancy of GpVI is not sufficient to elicit platelet activation. It is important to note that monomeric GPO6 that can accommodate at least two copies of GpVI lacks activity. Therefore, clustering of more than two copies of GpVI is required for platelet activation. If GpVI does indeed exist as a dimer at the platelet surface, then the data presented here may imply that clustering of three or more dimers, i.e. a complex comprised of at least six copies of the receptor, is the smallest GpVI cluster that can signal effectively.

Boyington and Sun (37), reviewing killer cell Ig-like receptor (KIR)-major histocompatibility complex (MHC) crystal structures, propose that, after ligation by MHC, KIR-KIR contacts provide a mechanism by which such leukocyte recep-
tor cluster (LRC) receptors might oligomerize, and so signal. The oligomeric GPO-containing peptides described here provide an extracellular array of sites that may complement the GpVI-GpVI contacts described by Horii et al. (13), and so stabilize the active receptor complex.

The reduction in protein tyrosine phosphorylation of platelets stimulated with 500 μg/ml compared with 50 μg/ml of monomeric CRP (Fig. 4) may reflect complete saturation of GpVI on the platelet surface, each receptor binding a single copy of CRP so that the clustering of receptors necessary for signaling becomes impossible, a phenomenon that is known in other systems (38).

Upon cross-linking, all peptides other than GPP10 and GPO1 acquire the capacity to signal, with Peptide GPO2-XL binding tightly enough to support sufficient receptor clustering and Peptides GPP4GPO2-XL combining lower affinity, measured as platelet binding, with a greater number of binding sites to achieve the same outcome. These two peptides are just above the activity threshold, failing to induce aggregation in a small proportion of donor platelets. All other peptides are fully active, albeit at quite high concentration. Previously, we have demonstrated that surface expression and functional responses of GpVI vary significantly between donors of different GpVI haplotype (39) and such differences may explain the variable levels of platelet adhesion or aggregation with these weaker peptide ligands.

In summary, the minimal functional triple helical recognition motif for the platelet collagen receptor GpVI contains two GPO triplets. These can be adjacent or spaced apart by four intervening triplets, although other spacings were not tested in this investigation. When adjacent, these provide a firm binding site for a single GpVI molecule, whereas, when spaced apart, each would bind to a separate GpVI molecule on the platelet surface. This allows the possibility that within the native collagen triple helix there are multiple potential binding sites for platelet GpVI, because there are five GPOGPO motifs within the α1 chains of both collagens I and III. Whereas there are over 30 single GPO triplets in each of these collagens, few of these are separated by only four intervening triplets, a spacing shown here to be GpVI reactive. Another important deduction is that monomeric collagens in solution will not, in general, activate platelets through GpVI (although a component of their interaction with the platelet is GpVI dependent), because a single triple helix such as Peptide GPO6 contains a longer stretch of contiguous GPO triplets than any native collagen sequence and does not support sufficient receptor clustering in non-cross-linked form. However, GpVI-reactive domains within the collagens may be identified by the density and separation of their GPO triplets, similar to the properties described here of Peptides GPP4GPO2 and GPP2GPO4. This will also involve detailed study of the relationship between GPO motifs in adjacent triple helices within a collagen fiber, because it seems probable that this will be the determinant of platelet activation through GpVI.

Further binding studies are required to ascertain which hydroxyproline-containing sequences in collagen are available to GpVI. A more precise description of the GpVI-collagen interaction will undoubtedly come from structural analysis of such molecular complexes.

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