Selective Blockade of Glycoprotein VI Clustering on Collagen Helices*

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Platelet activation by collagen relies on the interaction of the receptor glycoprotein V1 (GPVI) with collagen helices. We have previously generated two recombinant single chain human antibodies (scFvs) to human GPVI. The first, 10B12, binds to the collagen-binding site on the apical surface between the two immunoglobulin-like domains (D1D2) of the receptor and so directly inhibits GPVI function. The second, 1C3, binds D1D2 independently of 10B12 and has been shown to have a more subtle effect on platelet responses to collagen. Here we have shown that 1C3 potentiates the effect of 10B12 on platelet aggregation induced by collagen and cross-linked collagen-related peptide (CRP-XL). We investigated this by measuring the effect of both scFvs on the binding of D1D2 to immobilized collagen and CRP. As expected, 10B12 completely inhibited binding of GPVI to each ligand in a dose-dependent manner. However, 1C3 inhibited only a proportion of GPVI binding to its ligands, implying that it interferes with another aspect of ligand recognition by GPVI. To further understand the mode of inhibition, we used a unique set of CRPs in which the content of critical glycine-proline-hydroxyproline (GPO) triplets was varied in relation to an “inert” scaffold sequence of GPP motifs. We observed that a stepwise increase in D1D2 binding with (GPO)2 content was blocked by 1C3. Together these results indicate that 1C3 inhibits clustering of the immunoglobulin-like domains of GPVI on collagen/CRPs, a conclusion that is supported by mapping the 1C3 epitope to the region including isoleucine 148 in D2.

Recognition of exposed subendothelial collagen by the receptor glycoprotein V1 (GPVI)2 on platelets is a critical early step for platelet activation and subsequent thrombus formation. This interaction strengthens platelet adhesion through activation of integrins αIIbβ3 and α1β3 (1) and induces degranulation, aggregation, and procoagulant activity (2). Hence, elucidation of the mechanism of interaction of GPVI with collagen is valuable for developing effective platelet antagonists.

We have identified the primary collagen binding surface of human GPVI on the immunoglobulin (Ig)-like domains (D1D2) of the receptor by comparing binding of human and mouse recombinant monomeric D1D2 to a synthetic collagen-related peptide (CRP) that contains ten glycine-proline-hydroxyproline (GPO) repeats in each strand of a triple helix (3, 4). Prolyl hydroxylation is necessary for recognition of collagen and such peptides by platelet GPVI (5). Both mouse and human D1D2 showed specific, dose-dependent, and saturable binding to CRP, relative to peptide GPP10, a peptide of similar structure in which hydroxyproline is replaced by proline. Using such peptides, the minimum recognition motif for GPVI has recently been identified as a tandem GPO triplet or (GPO)_2.3

Using human recombinant D1D2 as bait, two scFvs were selected that bind to distinct epitopes on GPVI. 10B12 recognizes the primary collagen binding surface of human GPVI, and 1C3 does not (4). The 1C3 epitope in GPVI is as yet unknown but is conserved between human and mouse GPVI. 10B12 has been shown to inhibit collagen-induced platelet aggregation and prevent thrombus formation in vitro on a collagen-coated surface in whole blood perfusion (4). The effect of 1C3 on thrombus formation by human platelets under flow has also been investigated, where 1C3 was shown to alter neither the surface coverage nor the size and morphology of the thrombi formed on a collagen-coated surface. However, 1C3 halved the expression of phosphatidylserine on adherent platelets (7), leading us to conclude that 1C3 perturbs the function of GPVI indirectly, prompting the current investigation.

In this study the scFvs 10B12 and 1C3 are shown to have an additive inhibitory effect on collagen-induced platelet aggregation. The mode of receptor blockade conferred by 1C3 was investigated by measuring its effect on the binding of D1D2 to collagen and to synthetic collagen-like peptides. Also, evidence is presented that locates the epitope of the antibody to a surface of the molecule distinct from the primary collagen-binding site, providing an explanation for the biological effects of the antibody.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Division of Transfusion Medicine, Dept. of Haematology, University of Cambridge, Long Rd., Cambridge CB2 2PT, UK. Tel.: 44-1223-548095; Fax: 44-1223-548155; E-mail: pas28@cam.ac.uk. 2 The abbreviations used are: GPVI, glycoprotein VI; Ig, immunoglobulin; CRP, collagen-related peptide; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase.

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EXPERIMENTAL PROCEDURES

Materials—The anti-GPVI scFvs 10B12 and 1C3 were selected and characterized as described previously from human V gene phage display libraries (4). Human D1D2 (residues 1–185 of the mature protein) was cloned and expressed as a calmodulin-tagged fusion protein (4, 26) using full-length human GPVI cDNA and primers designed with reference to published sequences (8, 9). D1D2 has shown similar properties to human platelet GPVI (4). Horm collagen is a preparation of fibrillar type I collagen from equine tendon (Nymco, Munich, Germany). Collagen-related peptides are triple helices, each strand composed of 37 residues, and their synthesis has been described previously (10, 11). The primary structure of GPP10 is GCP-[GPP]_{10}-GCPG; GPO2 is GCP-[GPP]_{4} [GPO]_{4} [GPP]_{8}-GCPG; GPO4 is GCP-[GPP]_{3} [GPO]_{4} [GPP]_{3}-GCPG; GPO6 is GCP-[GPP]_{2} [GPO]_{6} [GPP]_{2}-GCPG; and monomeric (m)CRP is GCO-[GPO]_{10}-GCOG, where O = hydroxyproline. In CRP-XL, mCRP molecules were cross-linked via terminal cysteine or lysine residues as described previously (11). N9A peptide (CAARWKKAFIAVSAANRFKKS) (12) binds calmodulin in the presence of Ca^{2+} ions and was conjugated to BSA by a standard method (13) to give BSA-N9A. In this form it was used to immobilize D1D2 molecules in enzyme-linked immunosorbent assay (ELISA). To detect D1D2 binding to its ligands N9A was conjugated to peroxidase to give HRP-N9A. Protein purity was assessed by SDS-PAGE, and quantitation was performed using a BCA assay (Pierce).

Platelet Aggregometry—Citrate-anticoagulated whole blood was obtained from normal blood donors (National Blood Service, Cambridge, England) homozygous for the common allele (SKTQH) of the GP6 gene (14) and platelet-rich plasma (200 \times 10^9 platelets/liter) prepared as previously described (15).

Preparation of Monomeric and Dimeric scFvs—scFvs were expressed with c-Myc and hexahistidine affinity tags as previously described (16) and purified using a Ni^{2+}-charged HiTrap chelating column (Amersham Biosciences) according to the manufacturer’s instructions with 0.5 M NaCl, 50 mM sodium phosphate, pH 7.6–7.9, as the base buffer, eluting with 275 mM imidazole. This eluate contained a mixture of monomeric and dimeric (\approx 5\%) forms of each scFv. The latter species of 60 kDa likely represents a 1C3 diabody with two identical juxtaposed antigen-binding sites separated by 65 Å (17). After concentration (Vivaspin 10kDa; Vivascience) the scFvs were subjected to gel filtration (Superose 12; Amersham Biosciences GE Healthcare) into 10 mM HEPES, 150 mM NaCl, pH 7.2, to desalt and separate the two forms. These were kept on ice and used without delay to minimize re-equilibration.

Ligand Binding Assay for D1D2—Binding of D1D2 to collagen or collagen-related peptides was measured in a solid phase assay as previously described (4). Briefly, collagen or peptides were immobilized on 96-well MaxisorpTM microplates (Invitrogen). Equal coating of each peptide (GPP10, GPO2-GPO6, monomeric CRP) was ensured by measuring, in a separate experiment, the fluorescent signal obtained after derivatization of free cysteines of the peptides with Alexa 488-maleimide (Molecular Probes). Then, after appropriate blocking and washing, the binding of D1D2 was detected with HRP-N9A using a standard peroxidase substrate on a plate reader at 450 nm. Values for nonspecific binding to blocked wells (typically less than 0.06 absorbance units) were subtracted from other readings before analysis. Data were analyzed and graphs were produced in PRISM (GraphPad, San Diego, CA).

Inhibition Assay—The inhibition assay protocol is a modification of the standard ligand binding assay, with the addition of the following steps. A subsaturating concentration of D1D2 was preincubated in assay buffer either without any scFv or peptide (positive control of 100% binding) or in the presence of increasing concentrations of scFv or peptide for 2 h at room temperature before adding to the plate. Assay buffer was added to the remaining wells (negative control of 0% binding), and the plate was incubated for 20 min at room temperature. Bound D1D2 was detected as for the standard ligand binding assay. Specific binding was obtained by subtracting nonspecific binding (to the plate and/or BSA) from total binding.

Site-directed Mutagenesis of D1D2—Site-specific mutations were introduced into D1D2 using the QuikChange site-directed mutagenesis kit (Stratagene), essentially following the manufacturer’s instructions and as described previously (4). Residues Tyr-134 and Ile-148 were mutated individually to alanine using the following primer pairs: 5‘-GAAGGGGA-CCCTGGCCGGCAAAAGATCCCCAGAGATG-3‘ and 5‘-CCATCTCTCGGGATTCTTGGCGGGGCGAGGTCTTCCCTTC-3‘ for Y134A, 5‘-CCGGCTAGTTTCCCCATCCATCGGACCGGCC-3‘ and 5‘-CCGCGGTAGCGGTCAGCGAATGCAGAT-3‘ for I148A.

Capture ELISA—The effect of the single amino acid mutations on binding of D1D2 to the anti-GPVI scFvs 10B12 and 1C3 was investigated by ELISA. D1D2 mutants were captured via the calmodulin tag to BSA-N9A in the presence of Ca^{2+} and binding of the scFvs detected with an HRP-labeled mouse monoclonal anti-c-Myc (clone 9E10; Roche Applied Science) as described previously (4).

Structural Analysis of GPVI—The locations of Ile-148 and Tyr-134 on the surface of GPVI were determined using the crystal structure of the collagen-binding domains of GPVI (18). Prediction of likely protein interaction interfaces on the surface of GPVI was carried out using the Spipder web server (spipder.chcmc.org), which classifies amino acid residues based on their probability of forming protein interaction sites. The SPPI-DER1 algorithm was used, with the threshold value corresponding to the trade-off between sensitivity and specificity set to either 0.5 (the default value) or 0.7 (higher specificity). At both settings, Spipder identified a prominent patch of residues on the side of D2 adjacent to Ile-148 as a probable interaction site. Images were generated using PyMOL (20).

RESULTS

scFv 1C3 Potentiates 10B12 Inhibition of Platelet Aggregation Evoked by Collagen and CRP-XL—After separating the two forms of each scFv, the monomeric forms were used in these

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4 P. A. Smethurst, personal observations.
5 A. Porollo and J. Meiller, submitted for publication.
1C3 Epitope

Isoleucine 148 on a Side Face of GPVI Is Contained within the 1C3 Epitope—To further understand the inhibitory action of 1C3, we undertook to locate its epitope on GPVI using site-directed mutagenesis of D1D2. ScFvs 10B12 and 1C3 have been shown to bind D1D2 concurrently (4), indicating that 1C3 does not bind to the primary collagen binding surface recognized by 10B12, a conclusion confirmed by the data in Fig. 2. Previously published work shows that 1C3 does not bind to either Ig-like domain of GPVI when it is expressed individually, showing that both Ig-like domains are required for 1C3 binding (4). Also, biosensor experiments conducted in our laboratory show that the lectin derivative succinylated Concanavalin A binds to GPOGPO is not inhibited by scFv 1C3, whereas additional lat-

consistent with the dose range observed previously for binding to D1D2 by ELISA (4). The increase in binding produced by the 1C3 dimer most likely results from the increase in avidity of the bivalent scFv, which can link two D1D2 molecules together. It demonstrates that the epitope of this antibody is remote from the primary collagen-binding site, leading to the conclusion that 1C3 inhibits an interaction of D1D2 with CRP/collagen that is separate from the primary binding interaction.

scFv 1C3 Inhibits Clustering of D1D2 on Collagen-like Peptides with Increasing GPO Content—Binding of D1D2 to immobilized collagen-related peptides, GPO6, GPO4, GPO2, or the control GPP10, was measured in the ligand binding assay. Binding of D1D2 to these peptides increased relative to the GPOGPO content of each peptide as we have recently described.3 The effect of monomeric 1C3 on binding of D1D2 to peptides GPO6, GPO4, and GPO2 was then measured. A subsaturating amount of D1D2 (10 μg/ml) was preincubated with increasing concentrations of monomeric 1C3, then incubated in the peptide-coated wells, and subsequently bound D1D2 was detected. 1C3 reduced the binding of D1D2 to peptides GPO4 and GPO6 to the same level as binding to GPO2 (Fig. 3). Binding to the latter peptide was not diminished and was significantly above that to GPP10 across the range of scFv concentrations (one-way analysis of variance, p < 0.001). Hence, the direct interaction of GPVI with its minimal binding motif GPOGPO is not inhibited by scFv 1C3, whereas additional lateral clustering to adjacent motifs such as those present in peptides GPO4 and GPO6 is.

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the interdomain apex. In addition, 1C3 binds both human and mouse GPVI (4); therefore residues in the epitope are likely to be conserved between species. Further information on the localization of the 1C3 epitope came from a commercial peptide-based epitope-scanning screen of the Ig-like domains in which a series of overlapping 15-mer peptides covering the Ig-like domains of GPVI was synthesized and immobilized by a proprietary process (Pepscan Systems, Lelystad, Netherlands) and tested for reactivity with 1C3, 10B12, and irrelevant scFvs (acting as background controls). 10B12 reacted with peptides containing residues Lys-41 and Lys-59, consistent with previous work (3, 4). Three peptides were identified as likely to contain partial epitopes for 1C3, lying between residues Ser-144 and Ser-162 in the second Ig-like domain (data not shown). In the light of the above and information obtained from modeling, we designed and expressed two further single amino acid mutants of D1D2. Binding of 1C3 to wild-type and the two novel mutated forms of D1D2 was compared by ELISA. Each construct was immobilized via its calmodulin tag, ensuring equal loading and orientation of antigen. It was observed that, whereas binding of the scFv to Y134A and to wild-type D1D2 were of similar level, binding to I148A was completely abolished (Fig. 4A). When both mutants were tested for CRP binding activity, a slightly diminished binding was observed compared with that of wild-type D1D2 (Fig. 4B). However, binding was specific compared with the control peptide GPP10 (data not shown), indicating that the decrease in binding of 1C3 was not due to a general defect in D1D2 protein structure.

**DISCUSSION**

Activation of platelets by collagen is an important initial step in hemostasis and its pathological expression, thrombosis. GPVI is the major activating receptor on platelets for collagen and is a promising target for therapeutic intervention in atherothrombotic diseases (21). To facilitate the informed development of antagonists, it is important to understand the molecular details of the GPVI-collagen interaction. As both GPVI and collagen are insoluble, a number of molecular tools have been devised to investigate the GPVI-collagen interaction: recombinant soluble Ig-like domains of GPVI, termed D1D2, recombinant single chain variable domain antibody fragments, 10B12 and 1C3, selected on D1D2, and a series of synthetic triple-helical peptides that are structurally well defined and recognized by GPVI.

The primary collagen binding surface of human GPVI has been localized in this laboratory to the apical surface of the
Ig-like domains, using mutagenesis and binding studies (3, 4). In an independent study, non-biased in silico docking of CRP onto a 2.4 Å crystal structure of D1D2 identified the same interaction site (18), confirming our earlier studies. This collagen binding surface is also the epitope of the anti-GPVI scFv, 10B12 (4). Whereas 10B12 exerts clear inhibitory effects on all platelet responses to collagen (4, 7, 21), another anti-GPVI scFv, 1C3, selected against the same antigen, exhibited only a minor inhibitory effect on thrombus formation on immobilized collagen fibers and gave some reduction in procoagulant expression of adherent platelets, leading to the suggestion that 1C3 may exert its effect by interfering with receptor clustering on the extended collagenous ligand (4, 7).

In this study, 1C3 alone did not produce a significant functional effect on collagen and CRP-XL-induced platelet aggregation, in line with previous data (4, 7). However, in combination, 1C3 potentiated the response of 10B12, revealing its own more subtle inhibitory effect (Fig. 1).

Treatment of platelet suspensions with polymeric ligands of GPVI, such as CRP-XL or convulxin, triggers tyrosine phosphorylation of the Fc receptor H9253 chain and results in irreversible platelet aggregation (22–24). In contrast, monomeric forms of such agonists elicit a weak or no response (19, 25), leading to the conclusion that clustering of GPVI is critical for activation of the receptor. A binding mechanism involving dimeric GPVI was first proposed by Miura et al. (6) who used a dimeric Fc-GPVI construct. Very recently, the docking of multiple triple helices onto a dimeric GPVI has been proposed based on structural modeling (18); however, the precise mode of interaction of GPVI with collagen helices has remained biochemically uncharacterized.

To address this issue and to elucidate the functional behavior of scFv 1C3, we investigated the effect of the antibody on binding of D1D2 to CRP-XL, collagen, and a series of other GPO-containing peptides (Fig. 2, B and D). In sharp contrast to the partial inhibition observed for the 1C3 monomer, the 1C3 diabody greatly enhanced binding of D1D2 to both CRP-XL and collagen (Fig. 2, A and C). This may be explained by the dimeric 1C3-D1D2 complex having enhanced avidity for its ligands, which in turn demonstrates that 1C3 does not block the primary collagen-binding site. The inhibitory action of 1C3 must therefore depend upon a different feature of the GPVI-collagen interaction.

Because collagen is a complex heterogeneous molecule, for further binding studies we chose to use a series of well defined, triple-helical peptides in which the number of GPO triplets varied. By using D1D2 binding and platelet activation as readouts, the minimum GPVI binding motif has been proposed to contain two GPO triplets. Here it was observed that, in the presence of

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**FIGURE 4.** The effect of mutations on the activity of D1D2. A, epitope-mapping by ELISA. Wild-type D1D2 (wt) or the two point mutants (I148A, Y134A) were captured via the calmodulin tag to wells coated with BSA-N9A. The wells were incubated with scFv 1C3 (10 μg/ml), and then bound scFv was detected by 9E10-HRP. B, ligand binding activity. Binding of human D1D2 (squares), I148A (triangles), and Y134A (inverted triangles) to monomeric CRP-coated wells. Binding of calmodulin-tagged D1D2 was detected with N9A-HRP. Nonspecific binding to the control peptide GPP10 (e.g. 0.16–0.19 absorbance units at 300 μg/ml) was subtracted from each data point. In both experiments, values are representative of two separate experiments where the mean ± S.D. of three determinations is shown.

**FIGURE 5.** Three-dimensional structure of recombinant human D1D2 with significant residues highlighted. The crystal structure of D1D2 was solved as previously described (18). Ile-148 (red spheres) and Tyr-134 (gray spheres) are shown, together with surface residues implicated in binding to collagen/CRP (Lys-41, Lys-59, Arg-60, Arg-117, Arg-166). For reference, a docked model of CRP is illustrated to delineate the likely binding groove (18). Residues shown in green and cyan have been identified as probable protein interaction sites by the Sppider web server, using threshold values of 0.5 or 0.7, respectively.
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monomeric 1C3, binding of D1D2 to the peptides GPO4 and GPO6 was reduced to the same level as binding to peptide GPO2 (Fig. 3), providing direct evidence that 1C3 interferes with the ability of GPVI to cluster closely on these peptides.

This now elucidates the effect of the antibody on D1D2 binding to its polymeric ligands, CRP-XL and collagen. Binding to CRP-XL was reduced by ~80% in the presence of monomeric 1C3 (Fig. 2B). This is consistent with the binding of several D1D2 molecules per helix to adjacent sites in the absence of the antibody and the blockade of binding to those adjacent sites (but not the first on each helix) by 1C3. Binding to collagen is reduced by a lower proportion, ~50% (Fig. 2D), which is also consistent with the primary structure of collagen type I, which has a maximum run of two adjacent GPOGPO triplets in both α1 and α2 chains. We conclude that, in the presence of a saturating concentration of 1C3, GPVI receptors cannot cluster on adjacent binding motifs. Because platelet activation occurs even in the presence of 1C3 (4, 7), this implies that binding of GPVI to non-adjacent GPOGPO triplets on the same collagen chain, or to proximal GPOGPO triplets on other chains or collagen molecules, can induce signaling, although the magnitude of the response is reduced (7).

To visualize the effect of 1C3 on the receptor, its epitope was mapped by site-directed mutagenesis. The mutation of D1D2 I148A abolished the ability to bind 1C3 (Fig. 4A). In contrast, another hydrophobic mutation in the same Ig-like domain, Y134A, and numerous residues in the apical surface (3, 4) did not. These new mutations do not profoundly affect the structure of the protein, as they still bind specifically to CRP in a binding assay (Fig. 4B). An inspection of the position of Ile-148 in the recent structure of D1D2 (18) shows that the residue is present on a side face of domain 2 and is close to a putative protein interaction interface distal from the primary collagen-binding site (Fig. 5). From this, one can envisage how 1C3 may occupy an epitope separate from that of 10B12, straddling both domains and thus interfering with docking of GPVI to adjacent GPOGPO triplets in its ligands.

The work we present here using purified components will complement and inform other approaches that could be applied to study GPVI clustering, such as advanced microscopy. In conclusion, scFv 1C3 appears to be a valuable tool for distinguishing the effects of receptor clustering from direct ligand recognition on platelet activation by collagen through GPVI.

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