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Collagen–platelet interaction: Gly-Pro-Hyp is uniquely specific for platelet Gp VI and mediates platelet activation by collagen[1]

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Abstract

Objective: Peptides consisting of a repeat Gly-Pro-Hyp sequence are potent platelet agonists. The aim of this study was: (1) to examine the specificity of this sequence for platelet activation; (2) to confirm its recognition by platelet glycoprotein VI; and (3) to assess with suitable peptides the relative importance of glycoprotein VI and integrin α2β1 in platelet activation by collagen. **Methods:** Peptides were synthesized by standard Fmoc chemistry and tested for their ability to support adhesion of human platelets and HT 1080 cells, induce platelet aggregation, bind integrin α2 subunit A-domain and to cause tyrosine phosphorylation of platelet proteins. **Results:** (1) Peptides consisting of a repeat Gly-Pro-Pro, Gly-Pro-Ala or Gly-Pro-Arg sequence exhibited little if any platelet-reactivity. (2) The platelet-reactive peptide consisting of a repeating Gly-Pro-Hyp sequence failed to induce tyrosine phosphorylation in glycoprotein VI-deficient platelets. Platelet adhesion to this peptide was inhibited by intact anti-glycoprotein VI antibody and its Fab fragment. The latter inhibited aggregation by the peptide and fibres of both collagens I and III. (3) A peptide containing a 15-mer α2β1-binding sequence in a repeat Gly-Pro-Pro structure supported α2β1-mediated platelet and HT 1080 cell adhesion and bound α2 A-domain, but failed to activate platelets or to induce tyrosine phosphorylation. Conversely, a peptide containing this sequence but with an essential Glu replaced by Ala and inserted in a repeat Gly-Pro-Hyp structure did not recognize α2β1, but was highly platelet activatory. **Conclusions:** Platelet activation by collagen involves the highly-specific recognition of the Gly-Pro-Hyp sequence by platelet glycoprotein VI. Recognition of α2β1 is insufficient to cause activation. Interaction between collagen and glycoprotein VI is unique since Gly-Pro-Hyp is common in collagens but occurs rarely in other proteins, and glycoprotein VI may be expressed solely by platelets. This sequence could provide a basis for a highly-specific anti-thrombotic reagent to control thrombosis associated with plaque rupture.

Keywords: Human platelets; Thrombosis; Collagens; Collagen-based peptides; Integrin α2β1; Glycoprotein VI

1. Introduction

Rupture of the atherosclerotic plaque results in the exposure of circulating blood platelets to collagens in the plaque, notably collagens I and III, leading to platelet activation and thrombus formation, and culminating in sudden heart attack or stroke [1–3].

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[1] Amino acid sequences are defined using single-letter nomenclature (P*=Hyp); Ahx, 6-aminohexanoic acid; BSA, bovine serum albumin; CRP, collagen-related peptide; Fmoc, fluoren-9-ylmethoxycarbonyl; Gp, glycoprotein; mAb, monoclonal antibody; peptide-XL, crosslinked peptide; PRP, platelet-rich plasma.

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In earlier studies, we observed that collagen-related peptides (CRPs[1]), composed of a repeat Gly-Pro-Hyp sequence (GPP* using single-letter amino acid nomenclature; P*=Hyp) were highly platelet reactive [4]. Their reactivity was independent of the integrin α2β1, at that time the best established platelet collagen receptor [5]. CRPs induced platelet activation by the same mechanisms evoked by collagen, implicating the involvement of a common receptor [6–9]. We subsequently found that CRPs, like collagen, failed to activate glycoprotein (Gp) VI-deficient platelets suggesting that this receptor was Gp VI [10].

We present here new data demonstrating that the GPP* sequence is a highly specific platelet recognition sequence in collagen. We provide new evidence confirming that the receptor recognized by this sequence is Gp VI. We show too for the first time that recognition by α2β1 of specific α2β1-binding sequences in collagen is not sufficient in itself to cause platelet activation, in accord with the need for a second collagen receptor (Gp VI).

Brief accounts of some of this work have been reported elsewhere [11,12].

2. Methods

Collagen I for use in cell adhesion and solid-phase binding assays, and collagen III for use as an aggregatory agent, were prepared from calf skin by limited pepsin digestion as previously described [13,14]. Collagen III fibres were obtained by dialysis at 4°C of a solution of collagen III against sodium phosphate buffer, pH 7.6, ionic strength 0.02 [13]. A suspension of collagen I fibres obtained intact from bovine tendon was a gift from Ethicon, Somerville, New Jersey, USA, and was employed as a standard aggregatory agent as detailed previously [13,14].

The anti-(human integrin α2-subunit) mAb 6F1 [15] was a generous gift from Dr. B.J. Coller, Mount Sinai Hospital, New York, USA. Anti-Gp VI plasma [16] was gratefully received from Dr. H. Takayama, Graduate School of Medicine, Kyoto University, Japan. IgG was purified from the plasma by affinity chromatography on Protein A–Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) following the manufacturer's instructions. The Fab fragment was obtained from the purified IgG by digestion with immobilized papain (Pierce, Rockford, Illinois, USA) using Protein A–Sepharose to remove undigested IgG and Fc fragments. Control Fab was prepared in like manner from IgG isolated from normal human plasma. Recombinant integrin α2 subunit A-domain [17] was a kind gift from Dr. D.J. Tuckwell, School of Biological Sciences, University of Manchester, UK.

2.1. Cell adhesion assays

Platelet adhesion, at 20 or 37°C as specified, was measured using ^{51}Cr-labelled gel-filtered human platelets [13,14], or alternatively colorimetrically using washed human platelets [18]. Wells of Immulon 2 multi-well plates were coated with collagen I or peptide, routinely at a concentration of 10 μg/ml, for 1 h at 20°C. Adhesion was measured at 1 h. Results are expressed as the mean of triplicate determinations.

Adhesion of HT 1080 cells at 20°C was measured as described previously [19].

2.2. Platelet aggregation

Aggregation was measured turbidimetrically, at 20 or 37°C as required, using human citrated PRP, as previously described [13,14]. Aggregatory activity is expressed as the minimum agonist concentration required to give a full response. All assays included a standard, collagen fibres or CRP-XL, since we have observed that platelet responsiveness can vary considerably between individuals.

2.3. Integrin α2 A-domain binding

Binding of α2 A-domain to immobilized collagen or peptide was undertaken as described previously [17,20]. Briefly, bound A-domain fusion protein was detected using anti-glutathione S-transferase antibody, a peroxidase-conjugated secondary antibody, and 3,3′,5,5′-tetramethylbenzidine peroxidase substrate. Absorbance at 450 nm was measured using a multi-well plate reader.

2.4. Protein tyrosine phosphorylation

Tyrosine phosphorylation of platelet proteins in Gp VI-deficient platelets was measured as described by Ichinohe et al. [21]. Otherwise, the procedure of Hargreaves et al. [22] was employed. Briefly, following stimulation, platelet proteins were separated by SDS–polyacrylamide gel electrophoresis and, following blotting, detected with an anti-phosphotyrosine antibody and visualised by enhanced chemiluminescence.

2.5. Peptide synthesis

Peptides were synthesized as single chain C-terminal amides on TentaGel R RAM resin in a Perseptive Biosystems 9050 Plus PepSynthesizer by standard Fmoc protocols [23]. In general, Fmoc-amino acids (4 eq.) were activated with *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (4 eq.) in the presence of diisopropylethylamine (8 eq.). Fmoc deprotection was with a mixture of 2% (v/v) piperidine and 2% (v/v) 1,8-diazabicyclo[5,4,0]undec-7-ene. Peptides were released from the resin with trifluoroacetic acid, thioanisole, ethanedithiol and triisopropylsilane (90:5:2.5:2.5, by vol.). Crude peptides were purified by reverse phase HPLC on Vydac 219TP101522 with a linear gradient of acetonitrile in water. Fractions containing homogeneous product were

identified by analytical HPLC on Vydac 219TP54. The identity of the purified peptides was confirmed by mass spectrometry. Their spontaneous assembly into triple helices was demonstrated by determining melting curves by polarimetry [4].

The peptide $(GPA)_n$ was synthesized as a homotrimer covalently bonded at the C-terminus, by the method previously described [19]. Formation of a triple helix was demonstrated by polarimetry as with the other peptides.

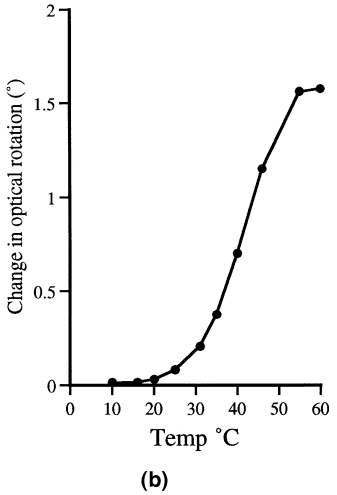
Peptides were crosslinked with 3-(2-pyridyldithio)-propionic acid *N*-hydroxysuccinimide ester as before [4,19].

3. Results

Peptides synthesized in this study are shown in Fig. 1a. All peptides were triple-helical at 20°C, the temperature at which assays were normally undertaken. Inclusion of a C residue at either end of the sequence allowed crosslinking to produce a polymer. Both triple-helical conformation and a polymeric structure are necessary for the expression of platelet-activatory activity [4,19].

PEPTIDE	STRUCTURE	$Tm_{1/2}$ (°C)
CRP	$GCP^*(GPP^*)_{10}GCP^*G\text{-}NH_2$	60
$(GPP)_n$	$GCP(GPP)_{10}GCPG\text{-}NH_2$	41
$(GPA)_n$	$GCA(GPA)_{14}GCA\text{—}Ahx$ $GCA(GPA)_{14}GCA\text{—}Ahx\text{—}K\text{—}K\text{—}Ahx$ $GCA(GPA)_{14}GCA\text{—}Ahx$	30
$(GPR)_n$	$GCR(GPR)_{10}GCRG\text{-}NH_2$	49
5/6-GAR	$GPC(GPP^*)_3GFP^*GARGVEGPP^*GPA(GPP^*)_3GPC\text{-}NH_2$	42
5/6-HYP2	$GPC(GPP)_5GFP^*GERGVEGPP^*GPA(GPP)_5GPC\text{-}NH_2$	44

(a)



(b)

Fig. 1. (a) Peptide nomenclature, sequences and melting temperatures. $T_{m1/2}$ is the temperature at which there is a 50% transition from helical to non-helical conformation. (b) Melting curve of peptide $(GPP)_n$ as a representative example; all peptides exhibited homogeneous transition curves.

3.1. Specificity of GPP as a platelet-reactive amino acid sequence in collagen*

Three novel peptides related to CRP were synthesized in this study and their platelet reactivity compared to that of the CRP. Each peptide was composed of a repeat triplet sequence, $(GXY)_n$, that was known to generate a triple helix when of sufficient length [24]. $(GPP)_n$, was identical to CRP except for the substitution of P* by P and spontaneously adopted a triple-helical conformation with a melting temperature ($T_{m1/2}$) of 41°C (Fig. 1b). To encourage triple-helical stability, $(GPA)_n$ was synthesized with fourteen repeat triplets and as a trimer covalently bonded at the C-terminus [24]. The melting temperature was 30°C. $(GPR)_n$ was identical to CRP except for the substitution of P* by R. As found by others [25], some days at 4°C were necessary for $(GPR)_n$ to adopt a triple-helical conformation. After two weeks at 4°, a melting temperature of 49° was recorded, indicating that the triple helix, once formed, was relatively stable.

In contrast to good adhesion to CRP [4], platelets showed no significant adhesion to $(GPP)_n$ and $(GPA)_n$ (Fig. 2), even when increasing the coating concentration to 500 μg/ml (data not shown). However, peptide $(GPR)_n$ showed variable adhesion. In four separate assays, adhesion to the peptide (at 20°C) was 30, 75, 75 and 130% of that to collagen. Within each assay, triplicate determinations were all within 10% of the mean.

After crosslinking, all three peptides had little if any aggregatory activity when tested at 20°C. Thus, whilst CRP-XL aggregated platelets at 5 ng/ml or higher, $(GPP)_n$-XL tested at the same time aggregated platelets only at 10 μg/ml and above, and was therefore 2000-fold less active than CRP-XL. $(GPA)_n$-XL was inactive when tested up to 1.5 mg/ml, whereas with the same PRP, CRP-XL was active at 100 ng/ml and higher, indicating that $(GPA)_n$-XL is at least 15 000-fold less active than CRP-XL. The peptide $(GPR)_n$-XL was 35 000-fold less active that CRP-XL, requiring 350 μg/ml versus 10 ng/ml required by CRP-XL for activity (results not shown).

3.2. Recognition of GPP by Gp VI*

3.2.1. Tyrosine phosphorylation in Gp VI-deficient platelets

Platelets exposed to CRP-XL exhibit a pattern of protein tyrosine phosphorylation broadly similar to that induced by collagen [6,8]. Collagen-induced phosphorylation of several proteins is severely impaired in Gp VI-deficient platelets, in accord with the lack of any aggregatory response of these platelets to collagen [21]. We show here (Fig. 3) that CRP, even at a concentration 100–1000-fold in excess of that required for the aggregation of normal platelets, is unable to induce any detectable tyrosine

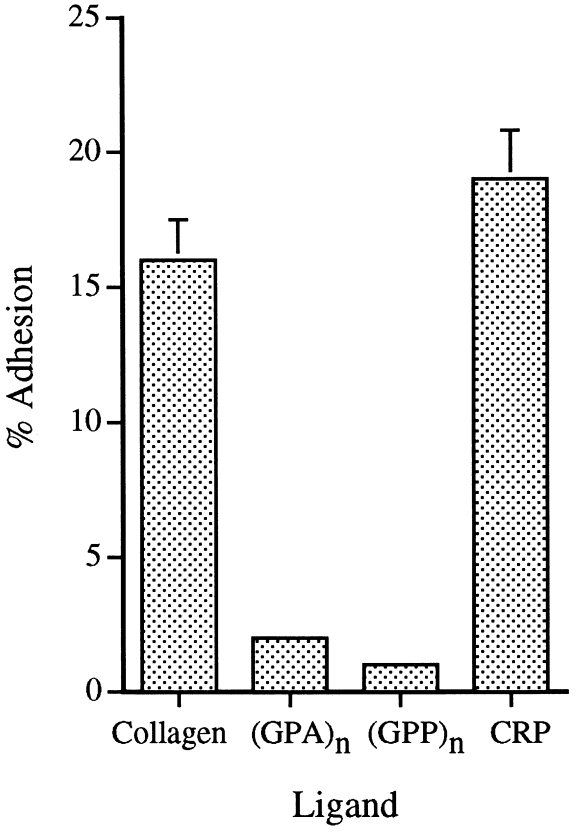


Fig. 2. Platelet adhesion to collagen, CRP and peptides $(GPP)_n$ and $(GPA)_n$. Adhesion at 20°C in the presence of 2 mM Mg^{2+} was measured at 1 h with ^{51}Cr-labelled platelets in 96-well plates. Collagen was bovine monomeric collagen type I. Wells were coated with collagen or peptide at 10 μg/ml. Assays were done in triplicate and the results are given as the mean±SD. Error bars, where not shown, were too close to reproduce. Data are representative of three similar experiments.

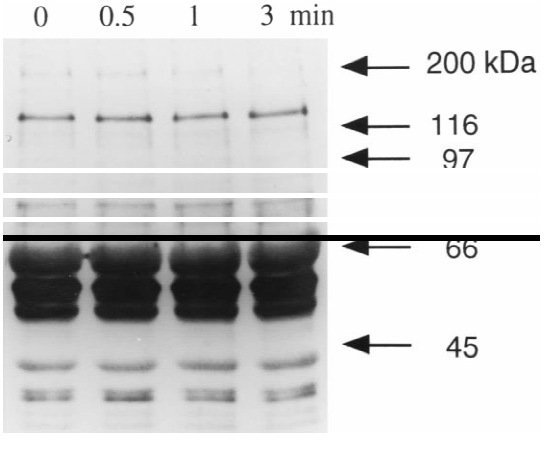


Fig. 3. Lack of CRP-induced protein tyrosine phosphorylation in Gp VI-deficient platelets. Platelets stimulated with CRP (10 μg/ml) for the times indicated were lysed, proteins separated by SDS–polyacrylamide gel electrophoresis and immunoblotted with an anti-phosphotyrosine mAb. Positions of molecular weight markers are shown.

phosphorylation above basal values in Gp VI-deficient platelets. In contrast, strong phosphorylation can be observed in normal platelets (see Fig. 9 by way of example). The lack of phosphorylation in Gp VI-deficient platelets confirms the crucial role of Gp VI as the activatory receptor recognizing both CRP and collagen.

3.2.2. Inhibition of the platelet reactivity of CRP by anti-Gp VI antibody

An anti-Gp VI antibody obtained from a patient with immune thrombocytopenic purpura has been reported to activate normal platelets, whilst the Fab fragment specifically blocks collagen-induced aggregation [26]. Here we report that both the intact antibody and the derived Fab fragment totally prevent platelet adhesion to CRP (Fig. 4), and that the Fab fragment strongly inhibits aggregation induced by CRP-XL and by fibres of collagens I and III (Fig. 5).

3.3. Collagen recognition of integrin α2β1 is insufficient alone for platelet activation

We have identified a sequence, GFP*GERGVEGPP*GPA, in the collagen I fragment α1(I)CB3, that on the basis of its ability to support α2β1-mediated cell adhesion and to bind purified α2β1 and the α2 A-domain, is an α2β1-recognition sequence [27]. Peptide 5/6–HYP2 (Fig. 1a) contains this sequence within a repeat GPP structure, rather than GPP* repeats used in the earlier study, to induce triple-helical conformation. The peptide binds the α2 A-domain (Fig. 6), and supports adhesion, as good as to collagen, of both HT 1080 cells and platelets that is divalent cation-dependent and strongly inhibited by anti-α2β1 antibodies (Figs. 7 and 8). Despite the recognition of α2β1, the cross-linked peptide 5/6-HYP2-XL is unable to induce detectable protein tyrosine phosphorylation (Fig. 9) or to induce platelet aggregation, even when tested at concentrations up to 2 mg/ml (data not shown).

Peptide 5/6-GAR (Fig. 1) contains the α2β1-recognition sequence in GPP* repeats except that the E in the GER triplet, essential for the recognition of α2β1, has been replaced by A so that the peptide no longer recognizes α2β1 and is unable to bind α2 A-domain [27]. The peptide fails to support adhesion of HT 1080 cells, which is dependent on the recognition of α2β1 [19] (Fig. 7). Platelet adhesion to the peptide occurs, but this is mostly cation-independent and is not affected by anti-α2β1 antibodies (Fig. 8). However, despite the lack of recognition of α2β1, the cross-linked peptide induces tyrosine phosphorylation comparable to that induced by CRP-XL or collagen (Fig. 9) and is highly platelet aggregatory, being able to induce aggregation at a concentration around 100 ng/ml (data not shown) and so is comparable in activity to CRP-XL, and more active than collagen fibres.

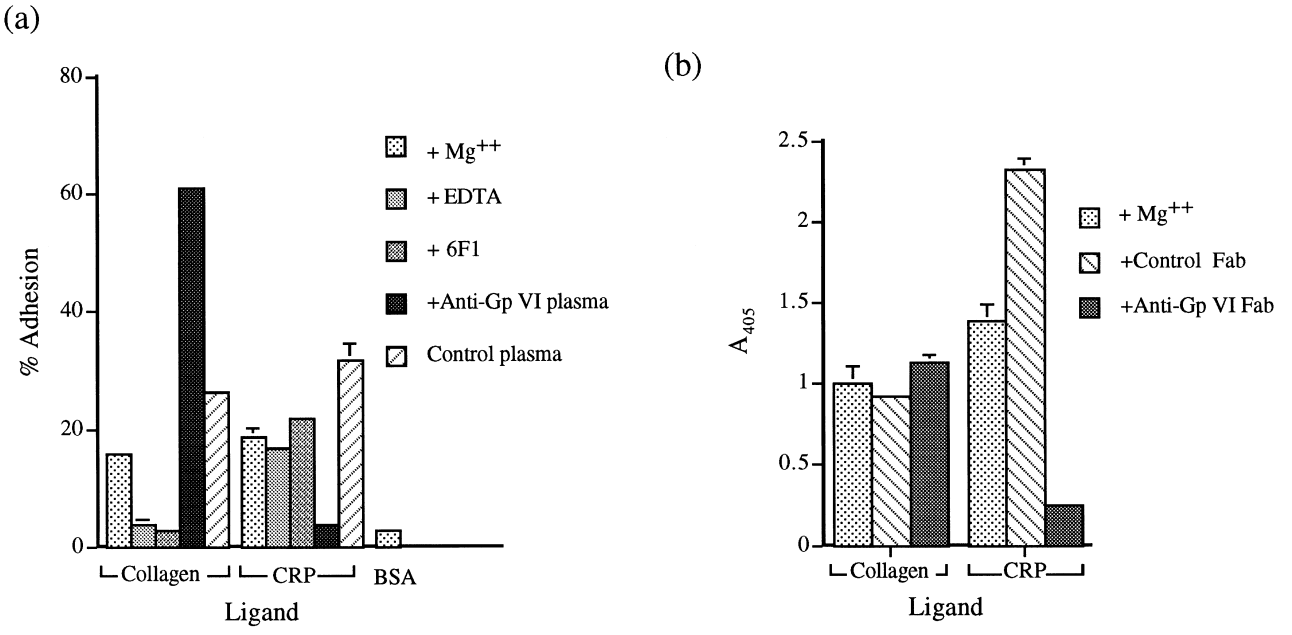


Fig. 4. Inhibition of platelet adhesion to CRP by anti-Gp VI antibody. (a) Adhesion at 37°C was measured in the presence of 2 mM Mg^{2+} except in the one instance where EDTA (2 mM) was used. Anti-Gp VI and control plasma were used at 10% (v/v), mAb 6F1 at 2 μg/ml. Other details are as in Fig. 2. Adhesion to collagen was blocked by 6F1, but stimulated by anti-Gp VI. Conversely, adhesion to CRP was unaffected by 6F1, but inhibited by anti-Gp VI. (b) Adhesion at 20°C in the presence of 2 mM Mg^{2+} was measured colorimetrically. Control and anti-Gp VI Fab were used at 200 μg/ml. Other details are as in Fig. 2. Anti-Gp VI Fab was without effect on adhesion to collagen but fully blocked adhesion to CRP. Results are given as the mean of triplicate determinations±SD Where error bars are omitted, they were too close to reproduce. Data are representative of two similar sets of experiments.

4. Discussion

*4.1. The specificity of GPP**

The failure of peptides $(GPP)_n$, $(GPA)_n$ and $(GPR)_n$ to exhibit any significant platelet activatory activity indicates that the potent reactivity of CRP is not attributable simply to its triple-helical conformation and that GPP* in collagen must represent a highly specific platelet recognition sequence. The inability of $(GPP)_n$ to support platelet adhesion or to induce significant aggregation, is especially remarkable given its close structural similarity to CRP. Platelet adhesion to $(GPR)_n$ may be due to a non-specific ionic interaction involving the multiple R residues, akin to

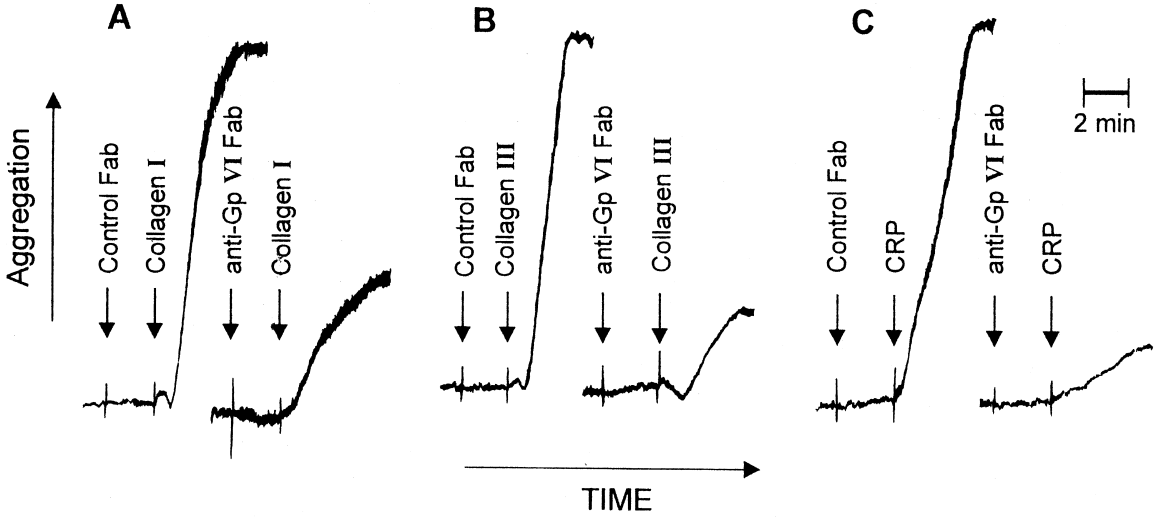


Fig. 5. Inhibition by anti-Gp VI Fab of platelet aggregation by collagen fibres and CRP-XL. (A) Collagen I fibres, 0.25 μg/ml; (B) Collagen III fibres, 10 μg/ml; (C) CRP-XL, 15 ng/ml. Concentration of agonist was the minimum required for full aggregation, measured at 37°C. Control and anti-Gp VI Fab were used at a final concentration of 250 μg/ml. The control Fab had no effect on aggregation (not shown). Anti-Gp VI Fab inhibited aggregation by the two collagens and by CRP. Data are representative of two similar experiments.

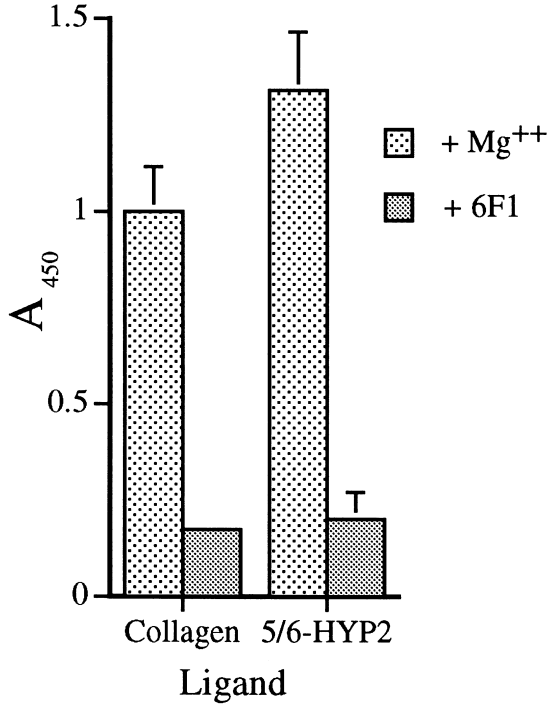


Fig. 6. Binding of integrin α2 A-domain to peptide 5/6-HYP2. Binding was measured in the presence of Mg^{2+} as detailed in Section 2. 6F1 was used at 2 μg/ml. Results are the mean of triplicate determinations±SD. Error bars are omitted where they were too close to reproduce. Data are representative of three repeat experiments.

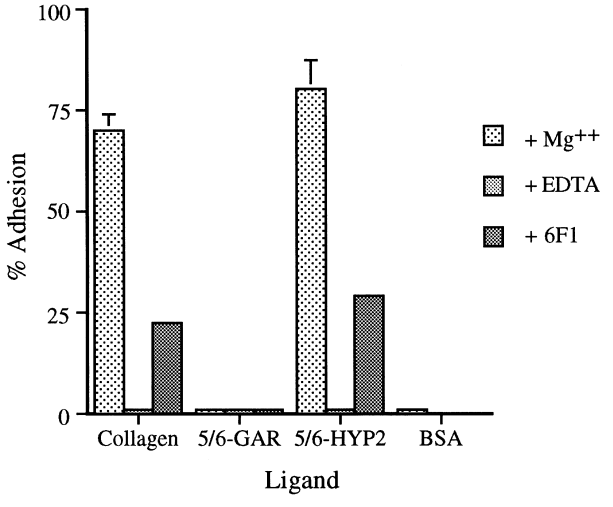


Fig. 7. Adhesion of HT 1080 cells to peptides 5/6-GAR and 5/6-HYP2. Adhesion, expressed as the number of adherent cells as a percentage of the total cell count, was measured at 20°C at 90 min in 96-well plates as described under Section 2. Collagen (bovine monomeric type I) and peptides were coated at 10 μg/ml. Adhesion was measured in the presence of 2 mM Mg^{2+} except where EDTA (2 mM) is indicated. 6F1 was used at 5 μg/ml. Results are the mean of triplicate determinations±SD. Where error bars are not shown, they were too close to reproduce. Data are representative of two similar experiments.

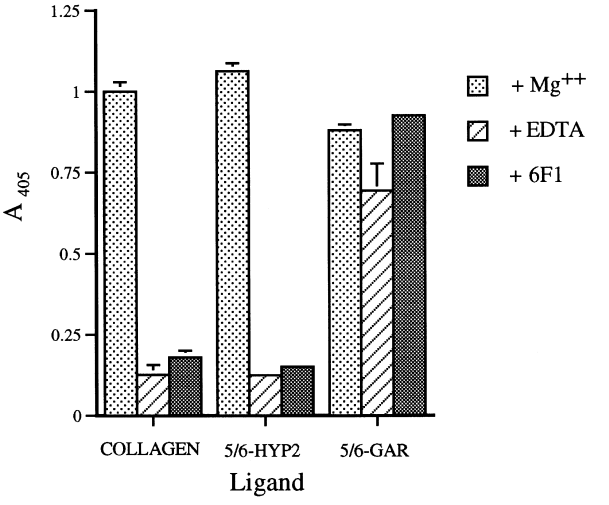


Fig. 8. Platelet adhesion to peptides 5/6-GAR and 5/6-HYP2. Adhesion at 20°C in the presence of 2 mM Mg^{2+} except where EDTA (2 mM) is indicated, was measured colorimetrically. 6F1 was used at 2 μg/ml. Other details are as in Fig. 2. In contrast to adhesion to 5/6-GAR, adhesion to 5/6-HYP2 is fully divalent-cation-dependent and fully blocked by 6F1.

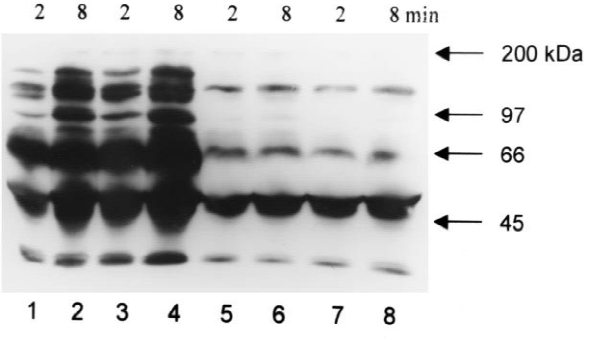


Fig. 9. Protein tyrosine phosphorylation in platelets stimulated by peptides 5/6-GAR and 5/6-HYP2. Tyrosine phosphorylation was measured as detailed in Fig. 4. Platelets were stimulated for 2 or 8 min with peptide (10 μg/ml) as follows: CRP, lanes 1 and 2; 5/6-GAR, lanes 3 and 4; 5/6-HYP2, lanes 5 and 6; vehicle, lanes 7 and 8. 5/6-GAR induces phosphorylation as strongly as CRP, whilst 5/6-HYP2 induces no phosphorylation above basal values. Similar results were obtained in a repeat experiment.

adhesion to polylysine. Variability of adhesion may reflect differences in platelet responsiveness between individuals, in the same way that this may also account for variations in the aggregatory activity of any given agonist.

4.2. Recognition of GPP by Gp VI*

The lack of ability of CRP to cause any tyrosine phosphorylation (above basal) in Gp VI-deficient platelets, and the inhibition of platelet adhesion to CRP and of CRP-induced platelet aggregation by anti-Gp VI Fab fragment, provides important new evidence that Gp VI is the primary receptor for CRP and that recognition of GPP* in collagen by Gp VI is essential for platelet activation, in

accord with our earlier proposal [10]. The intact anti-Gp VI antibody actually enhances adhesion to collagen (at 37°C), but this can be attributed to the fact that the antibody is activatory [26]. Attachment of the resultant aggregates to collagen mediated by α2β1 will in effect increase the number of adherent platelets. These same aggregates however will not attach to CRP since this requires the mediation of Gp VI and this is prevented by the antibody. Hence the intact antibody blocks adhesion to CRP.

The inhibition by anti-Gp VI Fab fragment of aggregation by collagen III, as well as collagen I, described for the first time here, indicates the need for Gp VI in platelet activation by both collagens. This is an important observation given the leading role of each collagen in platelet activation [2] and the evidence for specific receptors for each type [28].

4.3. The role of α2β1 in platelet activation

There is no doubt that α2β1 is an important platelet collagen receptor, being essential for platelet adhesion to collagen under flow conditions [1,5]. However, whether it has a role as a signalling receptor directly involved in platelet activation is unclear. There are reports of signalling in platelets emanating from α2β1 [8,21,29–36], but it is uncertain how much this reflects the role of α2β1 in facilitating platelet–collagen contact, so that the signals may in reality arise from an ancillary receptor such as Gp VI. Rather it may be that signals arising directly from α2β1 occupancy are primarily concerned with the adhesion process rather than with activation [36]. Our studies here have shown that peptide 5/6-HYP2 containing an α2β1 recognition sequence in a GPP repeat structure does not cause protein tyrosine phosphorylation in platelets and does not induce platelet aggregation. Conversely, peptide 5/6-GAR containing an inactive modified α2β1 sequence within a GPP* repeat structure, does not recognize α2β1, but is, nevertheless, highly platelet reactive. A number of conclusions can be drawn from these observations. The inactivity of 5/6–HYP2 and the potent activity of 5/6-GAR confirms the essential requirement for GPP* for platelet reactivity and that GPP is inactive. Secondly, it is clear that recognition by platelets of an α2β1 sequence in collagen presented in the native, triple-helical conformation is not sufficient in itself to cause platelet activation. However, since Gp VI-deficient platelets do not aggregate in response to collagen or CRP and since CRP acting through Gp VI and independently of α2β1 can induce aggregation of normal platelets, it can be concluded that Gp VI is both sufficient and necessary for platelet activation. Thirdly, the fact that adhesion to peptide 5/6-HYP2 is fully cation-dependent and totally blocked by the anti-α2 mAb 6F1 is convincing confirmation of the identification of GFP*GERGVEGPP*GPA as an α2β1 recognition sequence. In our previous work [27], this sequence was contained in a GPP* repeat structure and platelet adhesion to the peptide was only slightly impaired by 6F1, presumably due to GPP* itself supporting α2β1-independent adhesion via Gp VI. Our data here support a two-step model of collagen–platelet interaction [10,37,38] in which α2β1 is the important adhesive mechanism and Gp VI serves as the activatory receptor [9,10,16,21,37,39–41]. As yet though, we cannot rule out the possibility that some interplay or crosstalk between α2β1 and Gp VI contributes to the activation process, nor do we exclude a role for other collagen receptors [28,33,42].

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