Monomeric (glycine-proline-hydroxyproline)$_{10}$ repeat sequence is a partial agonist of the platelet collagen receptor glycoprotein VI

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We have previously reported that a triple-helical, collagen-related peptide (CRP; also known as CRP-XL) containing a glycine-proline-hydroxyproline (GPP*) repeat motif and cross-linked through cysteine residues at its N-terminus and C-terminus is a powerful stimulus of platelet aggregation and secretion through the surface receptor glycoprotein VI (GPVI). The activation of platelets is associated with tyrosine phosphorylation of the tyrosine kinase Syk and phospholipase C γ2 (PLCγ2). We now report that the non-cross-linked backbone of CRP, monomeric CRP (mCRP), stimulates the tyrosine phosphorylation of Syk and PLCγ2 in platelets and induces the weak secretion of [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) and aggregation. The action of mCRP does not seem to be due to spontaneous cross-linking, because alkylation of the cysteine residues leads to an increase in activity. The tripeptide backbone of CRP, GPP*$_{10}$ (in which P* represents hydroxyproline) also stimulates platelet shape change and the weak tyrosine phosphorylation of Syk and PLCγ2, but is unable to induce aggregation or secretion. The monomeric peptides partly inhibit the release of [^{3}H]5-HT by CRP, suggesting that they are partial agonists of the collagen receptor GPVI. These results demonstrate that GPP* present as a repeat motif is sufficient to activate the platelet collagen receptor GPVI but that the cross-linking of monomers brings about an increase in activity.

Key words: adhesion molecule, receptor recognition motif, tyrosine kinase.

INTRODUCTION

Collagen fibres become exposed at sites of damage to the vasculature, where they have a primary role in haemostasis through the regulation of blood platelets. Collagen fibres provide an important site of attachment (or adhesion) of platelets to the site of damage, leading to the deposition of a monolayer of cells over the exposed subendothelium. In addition, collagen fibres stimulate platelet activation, recruiting further platelets to the monolayer. The integrin $\alpha_2\beta_1$ is recognized as a major surface protein supporting the direct adhesion of platelets to collagen [1], but mounting evidence suggests that a second receptor, glycoprotein VI (GPVI), underlies activation (reviewed in [2,3]).

Evidence against a role for the integrin $\alpha_2\beta_1$ in platelet activation by collagen is provided by studies on a series of triple-helical, collagen-like peptides based on a GPP* repeat motif [4]. The prototype of this series, collagen-related peptide-cross-linked (CRP; also known as CRP-XL), has a backbone of GCP*(GPP*)$_{10}$GCP*G (single-letter amino acid code; P* represents hydroxyproline) which is cross-linked to yield a polymer through the N- and C-terminal cysteine residues. CRP is approx. 100-fold more potent than collagen in inducing platelet activation on a weight-for-weight basis but is unable to bind to $\alpha_2\beta_1$ [4–6]. The activation of platelets by CRP is inhibited in individuals deficient in GPVI [7] but is not altered in the presence of antibodies that prevent the binding of collagen to $\alpha_2\beta_1$ [4,6].

Collagen has recently been shown to activate platelets through the same pathway as that used by immune receptors [8,9]. Activation by collagen is associated with tyrosine phosphorylation of the Fc receptor (FcR) γ-chain, tyrosine phosphorylation and activation of the tyrosine kinase Syk and tyrosine phosphorylation of phospholipase C γ2 (PLCγ2) [8,10,11]. The tandem SH2 domains in Syk are believed to bind to the two phosphorylated tyrosine residues in the immunoreceptor tyrosine-based activation motif (ITAM) of the FcR γ-chain, leading to activation of the kinase. Essential roles for the FcR γ-chain and Syk in receptor signalling by collagen have been demonstrated by the loss of response in mouse platelets deficient in either protein [9]. Cross-linking GPVI by using a specific antibody [12,13] or through the snake venom toxin convulxin [14] stimulates the tyrosine phosphorylation of the FcR γ-chain and Syk, further supporting its role as a collagen receptor.

Monomeric CRP (mCRP) was shown to inhibit aggregation by collagen in platelet-rich plasma (PRP), suggesting that monomeric peptides serve as antagonists or weak partial agonists [4]. In the present study we have investigated the action of mCRP and various analogues on platelet activation and protein tyrosine phosphorylation to evaluate further their potential as receptor antagonists and to evaluate the requirement of quaternary structure for receptor activation.

MATERIALS AND METHODS

Materials

The following antibodies were used: anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology/TCS Biochemical, Botolph Claydon, Bucks., U.K.); anti-Syk rabbit polyclonal antibody Syk (LR) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). A suspension of type I collagen fibres from equine tendon was obtained as Horm collagen from Nycomed (Munich, Germany). The Src kinase inhibitor PP1 was purchased from

Abbreviations used: Abu-mCRP, 2-aminobutyric acid monomeric CRP; CRP, collagen-related peptide-cross-linked (also known as CRP-XL); FcR, Fc receptor; GPVI, glycoprotein VI; 5-HT, 5-hydroxytryptamine; m/aCRP, monomeric/alkylated CRP; mCRP, monomeric CRP; PLCγ2, phospholipase C γ2; PRP, platelet-rich plasma.

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Table 1 Sequence of CRP and its monomeric derivatives

The above peptides (single-letter code) were synthesized as described in the Materials and methods section. Abbreviations: Abu, 2-aminobutyric acid; NEM, *N*-ethylmaleimide; P*, hydroxyproline.

Peptide	Sequence
CRP	$(GCP^*(GPP^*)_{10}GCP^*G)_n$
mCRP	$GCP^*(GPP^*)_{10}GCP^*G$
m/aCRP	$GC(NEM)P^*(GPP^*)_{10}GC(NEM)P^*G$
GPP^*_{10}	$(GPP^*)_{10}G$
Abu-mCRP	$GAbuP^*(GPP^*)_{10}(GPP^*)GAbuP^*G$

Calbiochem. All other reagents were from sources described previously [6,15].

Synthesis of CRP and its monomeric analogues

mCRP was synthesized as described previously [4] and purified by preparative reverse-phase HPLC on diphenyl-Vydac [16]. Fractions containing the homogeneous product were identified by analytical HPLC and freeze-dried. The identity of the product was confirmed by matrix-assisted laser desorption ionization time-of-flight MS. CRP was made by cross-linking mCRP with 1.5 molar equiv. of *N*-succinimidyl 3-[2-pyridyldithio]propionate for 4 h at 21 °C in 0.1 M $NaHCO_3$. The cross-linked product was dialysed against 0.01 M acetic acid at 4 °C. The monomeric analogues of CRP, GPP^*_{10} and 2-aminobutyric acid monomeric CRP (Abu-mCRP) (see Table 1), were made, purified and characterized similarly. Alkylation of mCRP was performed as follows: mCRP (3 mg) in 0.1 M phosphate buffer, pH 6.0 (0.6 ml), was treated with tris(carboxyethyl)phosphine (1 mg) for 1 h at 21 °C. *N*-Ethylmaleimide (0.5 mg) in buffer (0.1 ml) was added and left to react for 1 h. The alkylated peptide was dialysed twice against 0.01 M acetic acid at 4 °C (1000 ml) and freeze-dried. Confirmation that alkylation was complete was by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) [17]; no free thiol was detected. Peptides were diluted in Tyrode's buffer before experimentation; the final concentration of acetic acid never exceeded 1 mM and in most experiments was no more than 1/10 of this.

Platelet preparation and functional studies

Experiments were performed on human platelets obtained from drug-free volunteers on the day of the experiment, as described [6]. Platelets were labelled with [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) in PRP as required. Platelets were isolated from PRP by centrifugation in the presence of prostacyclin (100 ng/ml) and suspended in a modified Tyrode's/Hepes buffer [134 mM NaCl/0.34 mM Na_2HPO_4/2.9 mM KCl/12 mM $NaHCO_3$/20 mM Hepes/5 mM glucose/1 mM $MgCl_2$ (pH 7.3)] at 4×10^8 cells/ml in the presence of EGTA (1 mM) and indomethacin (10 μM). EGTA and indomethacin were omitted from studies of aggregation unless stated otherwise. All experiments were performed at 37 °C in an aggregometer with continuous stirring (1200 rev./min) and were performed on at least three donors. Aggregation, secretion of [^{3}H]5-HT and protein tyrosine phosphorylation were measured as described [6]. Nonidet P40 (1 %, v/v) was used as the detergent in the immunoprecipitation studies [6].

Statistical analysis

All experiments were performed at least three times and results are shown as means ± S.E.M. unless stated. Statistical indication was by Student's *t* test; $P < 0.05$ was taken to be significant.

RESULTS

Functional studies

We have previously reported that the monomeric backbone of CRP, mCRP, is a weak antagonist of aggregation by CRP in PRP, demonstrating that cross-linking of the peptide is not required for receptor recognition and suggesting that analogues of mCRP might serve as antagonists. This has been explored in further detail in the present study through the characterization of the action of mCRP and several analogues (see Table 1 for structures) on platelet activation and protein tyrosine phosphorylation.

In contrast with the results observed in PRP [4], mCRP stimulated the full aggregation of washed platelets at a con-

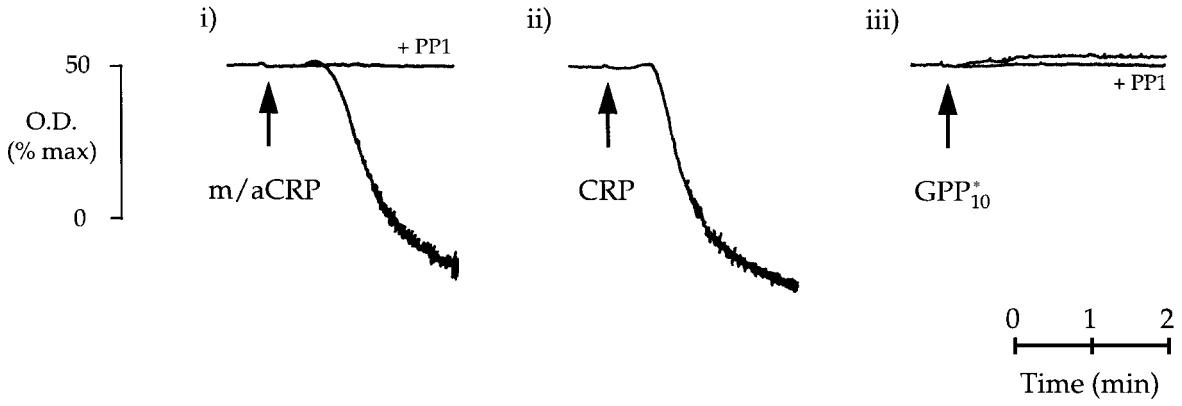

Figure 1 Studies on platelet aggregation

Platelets were resuspended in a modified Tyrodes-Hepes buffer at a density of 4×10^8/ml and aggregation was monitored by measuring the attenuance ('O.D.'). The Src-family kinase inhibitor PP1 (10 μM) was given 2 min before the peptide [m/aCRP (**i**), CRP (**ii**) or GPP_{10} (**iii**)]. Shape change is illustrated by an increase in attenuance; aggregation is illustrated by a decrease. Traces are representative of six to ten donors.

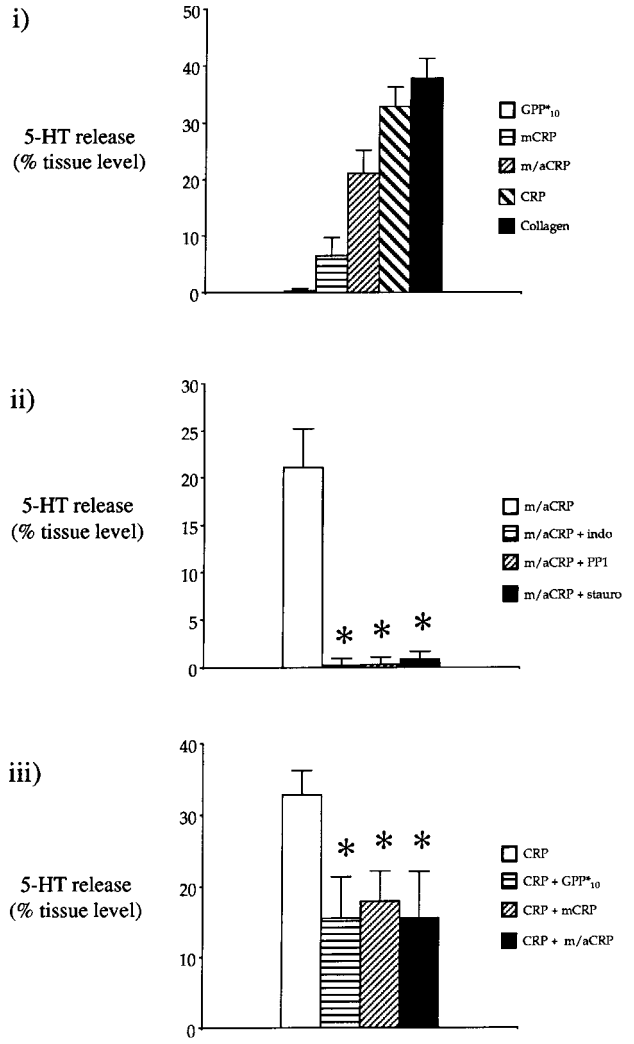

Figure 2 Studies on release of [^{3}H]5-HT

Platelets were resuspended, as described in the legend to Figure 1, after being prelabelled with [^{3}H]5-HT. All peptides were used at 100 μg/ml with the exception of CRP (3 μg/ml). The degree of release of [^{3}H]5-HT was measured as described in the Materials and methods section and is shown as a percentage of total tissue levels. Inhibitors were given 2 min before the peptide agonist and the incubations were left for a further 5 min. Results are means $\pm$ S.E.M. for four to seven experiments. *$P < 0.05$. (**i**) Stimulation of release of [^{3}H]5-HT by CRP and its monomeric derivatives; (**ii**) effect of inhibitors of tyrosine kinases (staurosporine and PP1, both at 10 μM) and of cyclo-oxygenase (indomethacin, 10 μM) on the release of [^{3}H]5-HT by m/aCRP; (**iii**) partial antagonist activity of monomeric peptides towards the release of [^{3}H]5-HT by CRP.

centration of 100 μg/ml, with shape change or partial aggregation observed at lower concentrations (10–30 μg/ml) (results not shown). Preparations of mCRP that had been stored at 4 °C for several days were noticeably more active, possibly owing to spontaneous cross-linking through the free cysteine residues. To address this possibility, mCRP was subjected to reduction with tris(carboxyethyl)phosphine and alkylation with *N*-ethylmaleimide. Monomeric/alkylated CRP (m/aCRP) induced full aggregation at 10–30 μg/ml (Figure 1). Alkylation of reduced mCRP with iodoacetamide brought about a similar increase in potency (results not shown). Abu-mCRP, in which 2-aminobutyric acid served as an isosteric replacement for cysteine, precluding the formation of disulphide bridges, was of similar activity to mCRP (results not shown). CRP induced full aggregation at 0.1–0.3 μg/ml, demonstrating that it is approx. 100-fold more potent on a weight-for-weight basis than m/aCRP. (The potency of CRP on a molar basis is far greater than this but cannot be measured because of its multimolecular structure.) These results demonstrate that cross-linking of mCRP is not essential for activation; however, cross-linking leads to a considerable increase in potency.

The greater activity of alkylated CRP relative to mCRP suggests a role for the N- and/or C-terminal regions of the molecule in the interaction with the receptor. We were therefore interested in determining whether the backbone of CRP, GPP*$_{10}$, retained affinity for the collagen receptor. GPP*$_{10}$ stimulated platelet shape change at 100–300 μg/ml (see Figure 1, for example) but did not stimulate aggregation at concentrations up to 8 mg/ml (results not shown). GPP*$_{10}$ (100 μg/ml) decreased the aggregation with 1 μg/ml CRP by up to 40 % in platelets from four of seven donors. One explanation for the inhibitory action of GPP*$_{10}$ is that it is a partial agonist of the receptor that underlies activation by CRP, namely GPVI.

The ability of the monomeric peptides to stimulate the release of dense granules was measured in platelets prelabelled with [^{3}H]5-HT. A maximally effective concentration of CRP (1 μg/ml) stimulated 34.4 $\pm$ 3.6 % release of [^{3}H]5-HT (Figure 2i). The response to collagen (30 μg/ml) was not significantly different from that to CRP, whereas the response to a maximally effective concentration of m/aCRP (100 μg/ml) was significantly smaller (Figure 2i). mCRP (100 μg/ml) stimulated a small degree of release of [^{3}H]5-HT but GPP*$_{10}$ was inactive (Figure 2i).

The importance of protein tyrosine phosphorylation in the response to m/aCRP was investigated by using two structurally distinct inhibitors of tyrosine kinases, the Src-family kinase inhibitor PP1 (Figure 1 and 2ii) and staurosporine, which are reported to block platelet activation by CRP [6,18]. Both inhibitors prevented aggregation, shape change and release of [^{3}H]5-HT by m/aCRP (Figure 1 and 2ii). Stimulation of shape change by GPP*$_{10}$ is also inhibited by PP1 (Figure 1) and staurosporine (results not shown). [^{3}H]5-HT release induced by m/a-CRP was dependent on cyclo-oxygenase activity (Figure 2iii).

The lower maximal release of [^{3}H]5-HT induced by the monomeric peptides in comparison with collagen and CRP is consistent with partial agonist activity. This was investigated by an examination of the ability of the monomeric peptides to decrease the response to CRP. mCRP, m/aCRP and GPP*$_{10}$ decreased the response to CRP to approx. 50 % of control values (Figure 2iii). The similar degree of inhibition observed with all three monomeric peptides was surprising in view of the different level of [^{3}H]5-HT released that was induced by each monomer on its own (Figure 2iii). This might reflect the combined effect of receptor antagonism and receptor desensitization, serving to bring about a similar level of inhibition by each peptide. Consistent with this, shorter periods of preincubation with m/aCRP cause less inhibition, possibly because of decreased receptor desensitization (results not shown).

Monomeric peptides stimulate tyrosine phosphorylation of Syk and PLCγ2

CRP stimulates the tyrosine phosphorylation of multiple platelet proteins including Syk and PLCγ2 (Figure 3). A similar pattern of tyrosine phosphorylation of platelet proteins, including Syk and PLCγ2, occurred after stimulation by mCRP, m/aCRP and GPP*$_{10}$ (Figure 3). The response to GPP*$_{10}$ was noticeably weak,

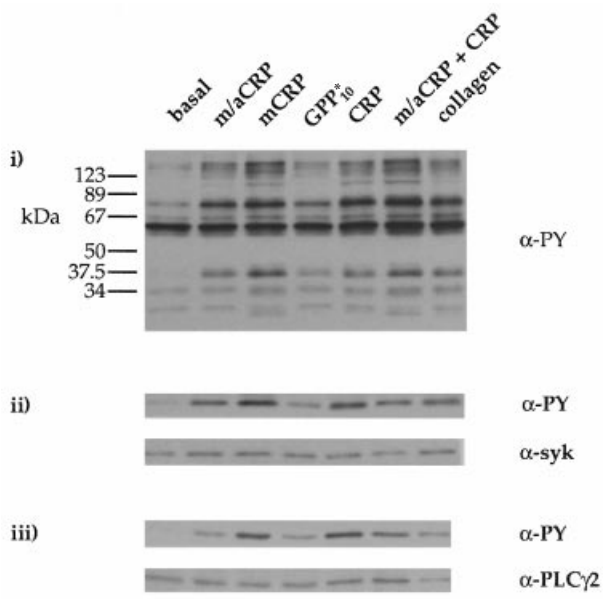

Figure 3 Tyrosine phosphorylation of platelet proteins including Syk and PLCγ2

Platelets were resuspended, as described in the legend to Figure 1, in the presence of EGTA (1 mM) and indomethacin (10 μM) and challenged with peptide agonists for 90 s while being stirred at 1200 rev./min. Experiments were stopped by transfer into sample buffer under reducing conditions. All peptides were used at a concentration of 100 μg/ml with the exception of CRP (3 μg/ml). Samples were taken for (**i**) analysis of whole-cell tyrosine phosphorylation by using the monoclonal antibody 4G10, (**ii**) analysis of tyrosine phosphorylation of Syk and (**iii**) tyrosine phosphorylation of phospholipase Cγ2 (PLCγ2) as described in the Materials and methods section. The upper panels in (**ii**) and (**iii**) show anti-phosphotyrosine (α-PY) blots and in the lower panels show reprobes for Syk (α-syk) and PLCγ2 (α-PLCγ2) respectively. The results are representative of four experiments.

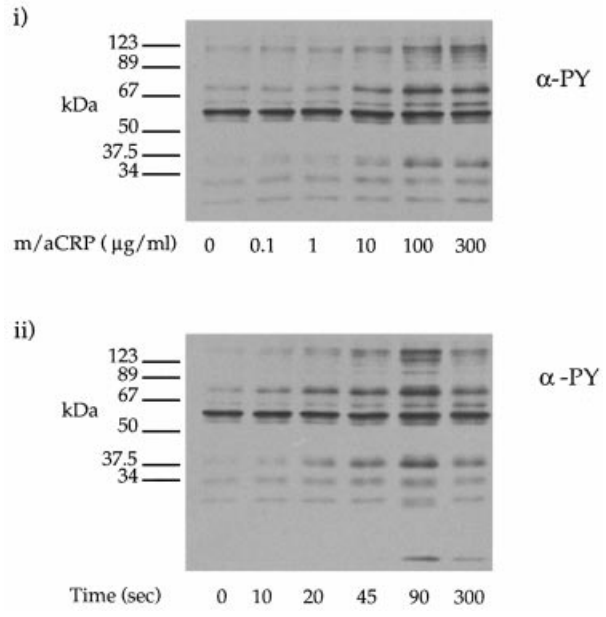

Figure 4 Concentration (i) and time-course (ii) relationships for stimulation of tyrosine phosphorylation by m/aCRP

Experiments were performed as described in the legend to Figure 3(**i**). The concentration of m/aCRP in (**ii**) was 3 μg/ml. Results are representative of three experiments.

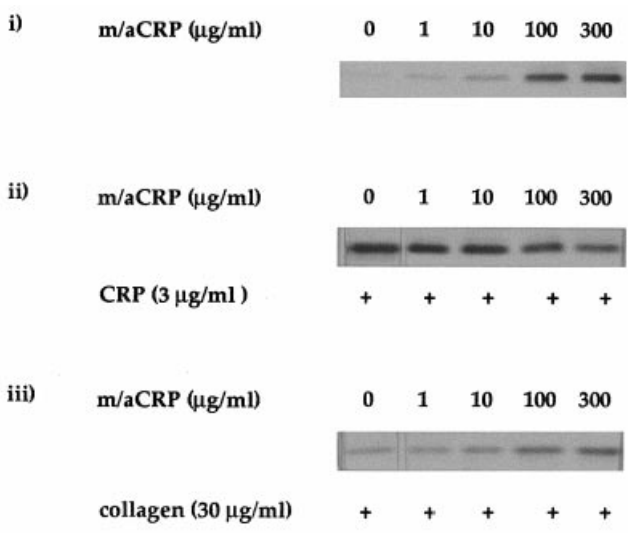

Figure 5 Tyrosine phosphorylation of Syk by m/aCRP in the presence of collagen and CRP

Experiments were performed as described in the legend to Figure 3. Syk was immunoprecipitated by using a specific antibody and measured for tyrosine phosphorylation by using the monoclonal antibody 4G10. A concentration–response curve for the phosphorylation of Syk by m/aCRP was determined either on its own (**i**) or in the presence of CRP (**ii**) or collagen (**iii**). m/aCRP was given 2 min before collagen or CRP. The results are representative of four experiments.

as clearly demonstrated by the small increase in tyrosine phosphorylation of PLCγ2 and the barely detectable increase in phosphorylation of Syk. m/aCRP induced a more pronounced increase in protein tyrosine phosphorylation, although the response was also lower than that to CRP. Moreover, when given together, m/aCRP decreased the tyrosine phosphorylation of Syk and PLCγ2 by CRP, suggesting a partial agonist activity of the monomeric peptide (Figure 3). mCRP also induced the tyrosine phosphorylation of multiple proteins, including Syk and PLCγ2 (Figure 3). The larger response to mCRP relative to that of m/aCRP in Figure 3 might have been due to a limited degree of cross-linking of mCRP caused by storage at 4 °C for several days, because the alkylated peptide was the more powerful stimulus in other studies.

Because of the variability in response to mCRP, further studies were performed with m/aCRP. Tyrosine phosphorylation of whole-cell proteins (Figure 4i) and of Syk (Figure 5i) by m/aCRP occurred at a threshold concentration of 1 μg/ml, with maximal activity at 100 μg/ml. The stimulation of tyrosine phosphorylation by m/aCRP occurred after a delay of 20 s and peaked at 90 s, at which time a tyrosine-phosphorylated band of 12 kDa was seen that co-migrated with the FcR γ-chain, the only tyrosine phosphorylated protein that has been shown to migrate in this region (Figure 4ii). The partial agonist activity of m/aCRP relative to CRP was explored in further detail by measurement of the tyrosine phosphorylation of Syk. m/aCRP decreased the level of tyrosine phosphorylation of Syk by CRP over the same concentration response range as that for which it stimulated phosphorylation (Figures 5i and 5ii), confirming partial agonist activity.

m/aCRP stimulated a similar or greater increase in the tyrosine phosphorylation of whole-cell proteins including Syk relative to collagen, suggesting that the latter is also a partial agonist (e.g.

Figures 3 and 5iii). This was investigated by monitoring the tyrosine phosphorylation of Syk after co-addition of the two agonists to platelets from a donor in which the response to collagen was significantly smaller than that to m/aCRP. Collagen decreased the degree of tyrosine phosphorylation of Syk by m/aCRP to a level intermediate between the responses to m/aCRP and collagen, suggesting that collagen has a lower efficacy than m/aCRP (Figure 5iii).

DISCUSSION

In the present study we have extended our studies on the structure–activity relationships required for the activation of the platelet collagen receptor GPVI. Previously we have shown that a triple-helical peptide, CRP, based on a GPP* repeat motif and cross-linked through cysteine residues at its N-terminus and C-terminus, is a potent and powerful activator of platelets [4–6]. Here we show that cross-linking of the monomeric backbone of CRP is not required for the activation of platelets and that GPP* presented as a repeat motif is sufficient to bring about the increase in protein tyrosine phosphorylation that is characteristic of platelet activation by CRP.

The close structural similarities of the monomeric peptides to CRP suggests that they are likely to mediate their effects through the same cell surface receptor, GPVI. Consistent with this, the monomeric peptides stimulate the same unique pattern of protein tyrosine phosphorylation as seen with collagen, including phosphorylation of the FcR γ-chain, Syk and PLCγ2; moreover, activation is blocked by tyrosine kinase inhibitors. Direct evidence that mCRP binds to GPVI is provided by two sets of observations: under static conditions, platelet adhesion to mCRP is inhibited by an intact anti-GPVI antibody and its Fab fragment [19]; secondly, mCRP inhibits the binding of the GPVI-selective radioligand I^{125}-convulxin [20,21] to platelets to a similar level to that seen by CRP (M. Leduc, M. J. Barnes and C. Bon, unpublished work).

The low potency and partial agonist activity of $GPP*_{10}$ relative to that of CRP demonstrates the importance of cross-linking in influencing agonist potency. A number of explanations might account for this result. CRP can be viewed as a series of monomeric peptides held together via cysteine residues. It is well established that multivalent ligands of this nature have a greater receptor affinity owing to their ability to bind simultaneously to several receptors on the cell surface. The lower efficacy of monomeric forms of CRP relative to CRP might also be due to the fact that the distance between GPP* repeat motifs is suboptimal. Inappropriate spacing between GPP* repeats might also explain the partial agonist activity of collagen relative to CRP and to m/aCRP. The sequence GPP* occurs a number of times in type I collagen but only up to five consecutive repeats. The cysteine residues and their surrounding residues in the C-terminus and N-terminus of CRP might also contribute to receptor affinity, either through direct binding or through an influence on the peptide conformation. The importance of substitutions in the terminal regions is highlighted by the increase in potency and activity of monomeric peptides with substitutions in this region relative to $GPP*_{10}$. The increase in activity is not dependent on the free cysteine residues or on spontaneous cross-linking because the replacement of cysteine by 2-aminobutyric acid retains activity, and alkylation by *N*-ethylmaleimide or iodoacetamide brings about a further increase in activity. Similarly, activity does not seem to depend on the self-association of peptides because no evidence for this was obtained during 8 weeks of storage of Abu-mCRP at 4 °C in Tyrode's buffer, as judged by laser light scattering (C. G. Knight and M. J. Barnes, unpublished work).

The above studies reveal a poor correlation between, on the one hand, the degree of tyrosine phosphorylation in platelets stimulated by collagen, CRP and m/aCRP and, on the other, the extent of [^{3}H]5-HT release. For example, collagen stimulates a degree of [^{3}H]5-HT release comparable to that induced by CRP (Figure 2i) but induces a significantly lower level of tyrosine phosphorylation of platelet proteins, including Syk and PLCγ2 (Figure 3) [6]. Similarly, tyrosine phosphorylation stimulated by collagen is similar to or less than that induced by m/aCRP, yet the alkylated monomer stimulates less [^{3}H]5-HT release. It is well established that thromboxanes have an important role in positive feedback in platelet activation by collagen. This is indicated, for example, by the inhibition of platelet activation by collagen in the presence of inhibitors of cyclo-oxygenase (results not shown). Similarly, the release of [^{3}H]5-HT and the stimulation of aggregation by m/aCRP are inhibited by the cyclo-oxygenase blocker indomethacin, whereas shape change is maintained (Figure 2, and results not shown). The poor correlation between the degree of tyrosine phosphorylation and the magnitude of [^{3}H]5-HT release by collagen and CRP might therefore reflect a differential role of thromboxanes in the response to the two agonists. An alternative explanation for the poor correlation is that other platelet-surface receptors potentiate the response to GPVI receptor activation by collagen but not by CRP or the monomeric peptides. For example, CRP is unable to bind to the integrin $\alpha_2\beta_1$ although little is known of its ability, or that of the monomeric peptides, to bind to other collagen receptors such as GPIV.

In summary, the present study has demonstrated that the sequence GPP* is sufficient to activate the collagen receptor GPVI when present in a repeat sequence; this is an important lead in the design of collagen receptor antagonists that have potential as novel anti-thrombotic agents.

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