Platelet Adhesion to Collagen and Collagen-Related Peptide Under Flow

Roles of the $\alpha_2\beta_1$ Integrin, GPVI, and Src Tyrosine Kinases

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Objective—Platelet stimulation by collagen and collagen-related peptides (CRPs) is associated with activation of protein tyrosine kinases. In the present study, we investigated the role of Src family tyrosine kinases in the initial adhesion events of human platelets to collagen and cross-linked CRP.

Methods and Results—Under arterial flow conditions, a glycoprotein VI–specific substrate, cross-linked CRP, caused rapid (<2 second) platelet retention and protein tyrosine phosphorylation that were markedly decreased by the Src family kinase inhibitor pyrozolopyrimidine (PP2) or by aggregation inhibitor GRGDSP. CRP-induced platelet retention was transient, and 90% of single platelets or aggregates detached within seconds. PP2, although having no effect on RGD peptide–binding to CRP, completely blocked aggregation and tyrosine phosphorylation of Syk and phospholipase C$\gamma_2$ (PLC$\gamma_2$). In contrast, PP2 weakly (<30%) suppressed firm adhesion to collagen mediated primarily by the $\alpha_2\beta_1$ integrin. Although PP2 prevented activation of Syk and PLC$\gamma_2$ in collagen-adherent platelets, tyrosine phosphorylation of several unidentified protein bands persisted, as did autophosphorylation of pp125$^{FAK}$.

Conclusions—These findings indicate that activation of Src-tyrosine kinases Syk and PLC$\gamma_2$ is not required for the initial stable attachment of human platelets to collagen and for FAK autophosphorylation. However, Src-tyrosine kinases are critical for glycoprotein VI–mediated signaling leading to platelet aggregation. (Arterioscler Thromb Vasc Biol. 2003;23:1934-1940.)

Key Words: platelets $\bullet$ adhesion $\bullet$ collagen $\bullet$ Collagen-related peptide $\bullet$ protein tyrosine kinase $\bullet$ flow

Platelet adhesion to collagens exposed during vascular injury is a primary step in the initiation of hemostasis. Several surface glycoproteins (GPs) have been proposed as platelet collagen receptors, including the $\alpha_2\beta_1$ integrin, 1 GP IV (CD36), 2 GPVI, 3 the type I collagen receptor (p65), 4 the type III collagen receptor (p68/72), 5 and 85- to 90-kDa GPs. 6 The $\alpha_2\beta_1$ integrin is involved in human platelet interaction with various collagens and is likely to be the major adhesion receptor under flow conditions, where strong interactions are essential to capture rapidly flowing platelets. However, it has been postulated that GPVI is critical for platelet adhesion to collagen. 10 Thus, mouse platelets in the absence of functional GPVI (FcR$\gamma$ chain deficiency) show impaired adhesion to collagen under static and flow conditions. 11 In agreement with this suggestion, $\alpha_2$- and $\beta_3$-deficient mouse platelets adhere to highly thrombogenic fibrillar collagen under conditions designed to suppress $\alpha_2\beta_1$ integrin function with similar kinetics and efficiency as wild-type platelets, indicating that the integrin receptor is not critical for adhesion. 12 In contrast, Chen et al 13 reported very poor adhesion of $\alpha_2$-deficient mouse platelets to collagen type I under static and flow conditions in the presence of Mg$^{2+}$.

A collagen-related peptide (CRP) is a synthetic peptide that mimics the triple-helical structure of collagen and, when stabilized by cross-linking, is a strong GPVI-selective agonist. 14-16 Because platelets adhere efficiently to CRPs immobilized on plastic surfaces in a static adhesion assay, it was thought that these peptides can be used for studying mechanisms of platelet adhesion to collagen under flow via the GPVI collagen receptor. 17 However, CRPs have been reported as unable to support platelet adhesion in whole blood under flow, possibly because of insufficient affinity to withstand shear forces without presence of other collagen receptors. 18 GPVI-deficient platelets in whole blood exhibit only a defective second phase of adhesion under flow conditions, suggesting that GPVI is involved in formation of platelet aggregates rather than in adhesion per se. 19

In contrast to reports about mouse platelets adhering to collagen in the absence of the $\alpha_2\beta_1$ integrin, there is strong evidence that firm adhesion of human platelets to various collagen types under flow conditions requires the $\alpha_2\beta_1$ integrin. 20 In the present study, we have investigated the mechanisms of the initial adhesion of human platelets to collagen...
collagen and to cross-linked CRP (CRP-XL) under flow conditions. Our findings are consistent with those obtained for the adhesion of human platelets and support a model in which the αiβ3 integrin plays a central role for stable anchoring of single platelets or aggregates to the collagen surface, whereas GPVI acts as a low-affinity coreceptor inducing strong aggregation but only transient and weak adhesion. In addition, although blockade of Src family of protein tyrosine kinases by the specific inhibitor pyrozolopyrimidine (PP2) had relatively little influence on collagen-induced adhesion, it completely suppressed CRP-induced tyrosine phosphorylation and αiβ3-dependent aggregation, suggesting that the initial firm adhesion via the αiβ3 is independent of GPVI-mediated tyrosine kinase activation.

Methods

Materials

Monomeric CRP (mCRP) peptide [GCO(GPO)6GCO] was provided by Drs Barnes and Farndale (Cambridge, UK) and was cross-linked through the C-terminal cysteinyl residues. Additional CRP was synthesized by our Biomolecular Research Facility (University of Virginia, Charlottesville, Va). Native type I collagen from rat skin was a gift from Dr Balian (University of Virginia). An anti-FAK mAb 2A7 was provided by Dr B. Coller (Mount Sinai Hospital, New York, NY). Anti-FAK mAb 2A7 and polyclonal IgG, BC3 were from Dr T. Parsons (University of Virginia). For additional information about antibodies and chemicals, please refer to the expanded Methods section in the online supplement, available at http://atvb.ahajournals.org.

Isolation of Human Platelets

Platelets were isolated from human blood by centrifugation in the presence of apyrase, indomethacin, and prostacyclin, washed, and finally resuspended in a modified fibrinogen-free Tyrode’s-HEPES containing 2 mmol/L Mg2+ with insoluble collagen fibrils (50 μg/mL) or with CRP-XL (0.5 μg/mL) and then processed for SEM analysis. Some experiments were performed in small aggregometer test tubes (Chronolog). For microscopy studies, platelets were continuously stirred for 30 seconds at 37°C in physiological buffer, more than 80% of bound platelets were flushed away during the 3 minutes. For additional information about antibodies and chemicals, please refer to the expanded Methods section in the online supplement.

Platelet Adhesion

The continuous-flow adhesion approach was as described.20 For additional information about rapid adhesion assay under flow conditions, please refer to the expanded Methods section in the online supplement.

Platelet Detachment, Immunoprecipitation, Immunoblotting, and Scanning Electron Microscopy

For platelet detachment assay, immunoprecipitation, immunoblotting, and scanning electron microscopy,21 please refer to the online supplement.

Statistical Analysis

All results are provided as mean±SE for the number of experiments indicated. Statistical significance was assessed by a single sample t-test, and in each case P<0.05 was taken to indicate a statistically significant difference.

Figure 1. Kinetics of platelet adhesion to CRP-XL and collagen under flow conditions. Platelets preincubated with buffer or GRGDSP (500 μmol/L) were pumped through Sepharose beads coated with collagen (coll I) or CRP-XL as described in Methods. Adhesion was determined by counting single platelets in the column effluent before and after adhesion and is expressed as the percentage of platelets attached to beads after subtraction of nonspecific adhesion to BSA-coated beads. The nonspecific adhesion to BSA was less than 5%. Values are expressed as mean±SE from 3 experiments.

Results

Transient Platelet Retention on CRPs Under Flow

The loss of single platelets trapped by CRP-XL immobilized on activated CH-Sepharose beads under flow conditions was rapid and reached maximal levels of 90% within 2 seconds (Figure 1). This platelet retention was strongly inhibited by 500 μmol/L GRGDSP, indicating that a sizeable proportion (approximately 65%) of platelet loss involved aggregation. Because a goal of our studies was to determine the contribution of CRP in adhesion, most subsequent experiments were performed in the presence of GRGDSP to prevent platelet aggregation.

A detachment assay (Figure 1 online) showed that peptide–independent platelet retention on CRP was weak and transient compared with collagen-adherent platelets. When the CRP-coated beads with attached platelets were flushed with a physiological buffer, more than 80% of bound platelets detached within 3 minutes. In contrast, only a small fraction of collagen-attached platelets (<3%) was flushed away during this time (Figure 1 online). SEM analysis revealed that in the absence of GRGDSP, the large platelet aggregates were weakly attached to the CRP-coated beads (Figure II online), because the washing and fixation steps removed them from the bead surface and the aggregates were then found on the filter surface used for SEM (Figure IIB online). In the presence of GRGDSP, only single platelets and small aggregates were attached to CRP-coated beads (Figure IIC online). Likewise, adhesion of these platelets was unstable, because they were lost rapidly from the beads and trapped on the filter surface (Figure IID online). Both the platelets attached to CRP beads and those found on the filter surface revealed significant shape change (Figures IIA through IID online). In contrast, collagen-adherent platelets were firmly attached (Figures IIE and IIF online) without noticeable shape change of the upper platelet surface.
Effects of the Tyrosine Kinase Inhibitor PP2 on Platelet Adhesion to CRP and Collagen

In the absence of GRGDSP, platelet deposition on CRP-XL beads after 90 seconds of perfusion was strongly (75% to 80%) blocked (Figure IIIA online) by the selective inhibitor of the Src family of protein tyrosine kinases PP2. In contrast, RGD-independent platelet binding to CRPs (approximately 23% to 35% of maximal retention) was insensitive to PP2. Preincubation with the combination of PP2, GRGDSP, and soluble mCRP almost completely suppressed platelet interaction with CRP-XL (Figure IIIA online). Neither GRGDSP nor soluble mCRP influenced platelet adhesion to collagen (Figure IIIB online). PP2 decreased platelet adhesion to collagen by less than 30% (Figure IIIB online).

To investigate in more detail the role of Src kinases in collagen-induced responses, we analyzed collagen- and CRP-induced platelet aggregation and adhesion by SEM (Figure 2). PP2 had no effect on platelet morphology in control resting platelets (Figures 2A and 2B). Platelets stimulated by CRP in suspension formed large aggregates (Figure 2C) whose formation was completely blocked by PP2 (Figure 2D). Resting platelets adhered efficiently to collagen-coated beads, generally forming a monolayer covering the bead surface without evidence of large aggregates during 90 seconds of perfusion (Figure 2E). Likewise, PP2-treated platelets adhered well to immobilized collagen and also formed a monolayer of adherent platelets on the collagen surface (Figure 2F). Platelets activated with collagen fibrils in suspension under stirred conditions revealed a shape change and formation of aggregates (Figure 2G), which were completely blocked by PP2 (Figure 2H). Nevertheless, the PP2-treated platelets adhered to collagen fibrils without undergoing shape change but formed characteristic necklace-like structures of single discoid platelets attached to collagen fibrils.

Protein Tyrosine Phosphorylation Induced by Adhesion to CRP and Collagen

In both static and effluent platelets (E) emerging from the collagen- or CRP-coated beads, several protein bands were tyrosine phosphorylated, including a 60-kDa protein band that migrated in the position of the Src tyrosine kinase and 67-, 80-, and the 130-kDa protein bands (Figures 3A and 3B).
Previously we showed that the shear forces experienced during flow through the collagen-coated beads or the addition of GRGDSP have no effect on protein tyrosine phosphorylation in effluent platelets compared with static controls.\textsuperscript{22} Adhesion to collagen was associated with an increase in tyrosine phosphorylation of several platelet proteins with approximate molecular masses of 80/86, 100/105, and 120/130 kDa (Figure 3A), and these increases were not influenced by GRGDSP (Figure 3A, right). Platelet retention on CRP beads was also associated with a large increase in the tyrosine phosphorylation of multiple protein bands (Figure 3B). However, although treatment with GRGDSP did not modify the pattern of tyrosine phosphorylation in effluent (E) platelets, it did strongly suppressed the major increases in tyrosine phosphorylation seen in CRP-retained platelets. Similar to other studies,\textsuperscript{16} there were few differences in the pattern of major tyrosine-phosphorylated proteins in collagen- and CRP-bound platelets. The effect of PP2 on overall tyrosine phosphorylation and phosphorylation of downstream molecules such as Syk, phospholipase C\textsubscript{\gamma2} (PLC\textsubscript{\gamma2}), and FAK in collagen and CRP-bound platelets was also examined. PP2 had a profound effect on tyrosine phosphorylation levels in effluent (E) and static platelets, causing major decreases. PP2 completely prevented the increases in tyrosine phosphorylation of platelets deposited on CRP beads (Figure 3B) without changing the RGD-insensitive binding.

The presence of PP2 resulted in very different effects on adhesion and tyrosine phosphorylation in platelets adherent to collagen (Figure 3A, left) compared with CRP (Figure 3B). The overall protein tyrosine phosphorylation was substantially reduced by PP2, whereas phosphorylation of several bands of 53, 58, 60, 105, 120, and 130 kDa remained, and their relative increases were still significant compared with static controls exposed to PP2 (Figure 3A, left). Additional tests were performed to identify proteins that became tyrosine phosphorylated even in the presence of PP2. PP2 completely prevented tyrosine phosphorylation of Syk and PLC\textsubscript{\gamma2} induced either by adhesion to collagen or retention on CRP (Figures 4A and 4B).\textsuperscript{18} Independent activation of FAK is observed both in platelets adherent to collagen under flow\textsuperscript{22} and in platelets stimulated with collagen fibrils in suspension.\textsuperscript{25} In contrast, platelet binding to CRP when aggregation was prevented did not induce FAK activation (Figure 4C, right). Tyrosine phosphorylation of FAK in collagen-adherent platelets was partially blocked by PP2 (Figure 4C). To examine the role of Src family kinases in adhesion-mediated phosphorylation of FAK, the effect of PP2 on FAK autophosphorylation at Tyr-397 was determined. Platelet treatment with PP2 did not interfere with collagen-induced FAK autophosphorylation, as shown by Western blotting with an antibody directed against Tyr(P)-397 of FAK (Figure 4C). In contrast, PP2 completely blocked tyrosine phosphorylation of FAK in CRP-aggregating platelets. Similar amounts of FAK were recovered after treatment with or without PP2 (Figure 4C, bottom).

**Discussion**

**Tyrosine Phosphorylation During Adhesion to Collagen and CRP**

Platelets were rapidly trapped/retained on CRP immobilized on CH-Sepharose beads under flow conditions in the presence or absence of an inhibitor of aggregation (Figure 1). However, unlike adhesion to collagen, the binding was weak and transient, causing increased tyrosine phosphorylation in the attached platelets, whereas effluent cells revealed no phosphorylation changes (data not shown). Inhibition of platelet aggregation reduced retention by more than 60% (Figure IIIA online) and sharply lowered tyrosine phosphorylation (Figure 3B). Thus, the large increase in tyrosine phosphorylation can be attributed to aggregation rather than to binding of individual platelets to CRP. Interestingly, the substantial tyrosine phosphorylation and aggregation induced by CRP (Figure 3B) did not enable firm platelet attachment (Figures I and II online), suggesting that intracellular signaling generated via CRP binding to GPVI is not sufficient to support firm adhesion either of single platelets or aggregates under flow conditions. Therefore, our results confirm that peptide sequences in collagen other than triple helical structure repeat present in CRP are critical for the firm platelet adhesion to collagen under physiological flow conditions.\textsuperscript{18}

**Roles of Src-Related Kinases in Platelet Adhesion**

Src family kinases are involved in intracellular signaling mediated via integrins in response to cell adhesion to extracellular matrix proteins\textsuperscript{26} and via immune receptors, leading to Syk activation.\textsuperscript{27} Human platelets express high levels of...
Src, Fyn, Hck, Lyn, Yes, Lck, and Fgr tyrosine kinases. The selective Src family kinase inhibitors PP1 and PD173956 have been shown to block collagen- and CRP-induced shape change, secretion, aggregation, and tyrosine phosphorylation, suggesting that Src-related kinases are of major importance in platelet stimulation by collagen. In agreement with these studies performed under low-shear stirring conditions, we have found that under arterial flow the Src family kinase inhibitor PP2 completely blocked CRP-induced aggregation and shape change without affecting the initial platelet binding to immobilized CRP (Figure IIIA online). Although Src kinase activity was not required for initial, transient platelet binding to CRP, it was essential for subsequent GPVI-mediated signaling that led to aggregation and dense granule secretion (data not shown). In the absence of aggregation, CRP induced tyrosine phosphorylation of Syk and PLCγ2 (Figures 4A and 4B) but not FAK (Figure 4C). In contrast to our findings, Achison et al. reported that CRP could stimulate FAK activation in the absence of aggregation probably via direct stimulation of GPVI. It remains unclear whether these differences in the requirements of α5β1 engagement in FAK activation by CRP represent different effects of soluble and immobilized ligand. Inhibition of Src kinase activities suppressed tyrosine phosphorylation of Syk and PLCγ2 without affecting the initial platelet interaction with CRP. This suggests that GPVI binding to CRP occurred without activation of Syk and PLCγ2 and that Src kinases acting upstream of Syk and PLCγ2 were not required for GPVI-mediated platelet binding to CRP. PP2 was added at concentrations previously shown to be specific for Src kinases and used to demonstrate a role of these kinases in collagen-induced aggregation and secretion.

Surprisingly, the Src kinase inhibitor PP2 had little effect on platelet adhesion to collagen, and in strong contrast to CRP-bound platelets, PP2 did not block completely the increases in tyrosine phosphorylation seen in collagen-adherent platelets (Figure 3A). Nevertheless, tyrosine phosphorylation of Syk and PLCγ2 induced by adhesion to collagen was blocked by PP2 (Figures 4A and 4B), indicating that these enzymes were not required for firm rapid adhesion to collagen. Tyrosine phosphorylation of FAK was only partially prevented by PP2, and FAK autophosphorylation on Tyr-397 was unchanged compared with controls. This suggests that the CRP binding site on GPVI was not involved in the signaling pathway leading to FAK activation (Figure 4C).

A limited role for Src kinases in firm adhesion is at first sight surprising. However, focal adhesions and cell adhesion are relatively unaffected in fibroblasts isolated from Src-, Fyn-, or Yes-deficient mice. In addition, normal cell adhesion can be restored by a mutant c-Src lacking the kinase and C-terminal domains, suggesting that recruitment of other proteins mediated by the SH2 and SH3 domains of c-Src is important for cell adhesion. It could be that kinase-dependent effects are supplied by remaining Src kinases in Src-deficient cells but specific SH2 and SH3 interactions with other proteins are also required. These experiments do not rule out roles for Src family kinase activity. Loss of Src expression may not affect integrin-ligand interactions, whereas it could influence the strength or dynamics of integrin-cytoskeleton interactions. Thus, our results are consistent with the hypothesis that the tyrosine kinase activity of c-Src is not needed for the initial adhesion and for autophosphorylation of FAK, but rather that Src-related kinases may have a role in platelet spreading and adhesion-induced activation of the fibrinogen receptor. Indeed, inhibition of Src kinases by PP1 blocks both platelet spreading on collagen and activation of Rac and PAK. The findings that initial stable platelet attachment to collagen did not require activation of Src upstream of Syk and PLCγ2 (Figures 4A and 4B) suggest that the Src kinases are not involved in the initial adhesion events and activation of αβ integrin.

Collagen Receptors Involved in Signaling During Adhesion

One of the aims of our study was to evaluate the role of GPVI collagen receptor in platelet adhesion under flow conditions. Previously, we have shown that platelet adhesion to native type I collagen is mediated primarily via the αβ integrin in a Mg2+-dependent manner without causing immediate shape change and release of dense-granule contents. This rapid adhesion in the absence of plasma proteins favors αβ integrin and does not require platelet GPIb and von Willebrand factor (vWF), because it is neither blocked by anti-GPIb antibody SZ2 nor is it increased by soluble vWF (data not shown). In contrast, immobilized CRP-XL induced rapid shape change and α-granule secretion that were not blocked by GRGDSP (data not shown). Thus, vWF could be rapidly release from α-granules and exposed on platelets bound to CRP. Flowing platelets that express GPIb receptor would bind to this secreted vWF, forming aggregates that are insensitive to RGD and Src kinase inhibitor and easily removed from CRP-coated beads under flow. There is evidence that platelet interaction with collagen via the αβ integrin induces signal transduction events associated with activation of FAK and PLCγ2. However, it is not clear how far the integrin is directly responsible for signaling leading to platelet activation, because anti-αβ antibodies neither block tyrosine phosphorylation nor activation of FAK induced by fibrillar collagen, when aggregation rather than adhesion is dominant.

Our new results suggest that GPVI-mediated signaling sensitive to PP2 is not involved in the initial adhesion to collagen but rather is important for subsequent platelet aggregation and thrombi formation. Platelets exposed to PP2 at concentrations that blocked CRP-XL-induced tyrosine phosphorylation...
phosphorylation and aggregation adhered efficiently to collagen and even showed significant tyrosine phosphorylation (Figures 3A and 4C). Because the anti-α2β1 antibody 6F1 blocks most of the rapid adhesion, the α2β1 integrin seems to play a critical role in forming the initial attachment to collagen.22 Thus, our results support a model of platelet interaction with collagen where the initial binding via α2β1 integrin serves to bring the collagen into the vicinity of a second receptor, most likely GPVI, which subsequently mediates tyrosine kinase activation that leads to aggregation.

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References

32. Kaplan KB, Bibbins KB, Swedlow JR, Arnaud M, Morgan DO, Varmus HE. Association of the amino-terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527. EMBO J. 1994;13:4745–4756.


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