Proteolytic Cleavage of the $\beta_1$ Subunit of Platelet $\alpha_2\beta_1$ Integrin by the Metalloprotease Jararhagin Compromises Collagen-stimulated Phosphorylation of pp72$^{\text{syk}}$*

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Aura S. Kamiguti‡§, Francis S. Markland¶, Qing Zhou¶, Gavin D. Laing¶, R. David G. Theakston, and Mirko Zuzel¶

From the ‡Department of Haematology, Royal Liverpool Hospital, University of Liverpool, Liverpool L69 3BX, United Kingdom, §Department of Biochemistry and Molecular Biology, University of Southern California, Los Angeles, California 90033, and ¶Alistair Reid Venom Research Unit, Liverpool School of Tropical Medicine, Liverpool L3 5QA, United Kingdom

Activation of platelets by different adhesive proteins involves binding of these proteins to integrins. Integrins belong to a superfamily of heterodimeric (αβ) cell surface glycoproteins that participate in cell-cell and cell-matrix interactions (1). Following adhesion, platelet activation is marked by the characteristic features of shape change, granule secretion, and aggregation, involving mechanisms which are not entirely understood. However, it is well established that these effects are accompanied by tyrosine phosphorylation of a number of proteins, demonstrating a role of tyrosine kinases in integrin-induced stimulation signaling (2–6). The understanding of the precise sequence of events involved in collagen-induced signaling in platelets is further complicated by the presence of multiple collagen-binding receptor proteins on these cells. Thus, the platelet/collagen interaction is thought to be mainly mediated by the Mg$^{2+}$-dependent $\alpha_2\beta_1$ integrin (7–9) but also involves the $\alpha_1\beta_1$ integrin (10), glycoprotein IV (CD36) (11), glycoprotein VI (12), and some 85- to 90-kDa glycoproteins not yet characterized (13). The proteins which become tyrosine phosphorylated upon platelet stimulation by collagen include the Fc receptor γ-chain (14), the low affinity immunoreceptor FcγRII (15), the nonreceptor tyrosine kinase pp72$^{\text{syk}}$ (5, 16, 17), and phospholipase C_2 (15, 18–20). The precise role of these different proteins in collagen-induced signaling via different platelet collagen receptors has been the subject of intense recent investigation (14, 15, 21).

Poor platelet response to fibrillar collagen with decreased protein tyrosine phosphorylation including that of pp72$^{\text{syk}}$ is associated with deficiencies of the membrane glycoprotein VI (12, 22, 23) and $\alpha_2\beta_1$ integrin (24–26). Platelet $\alpha_2\beta_1$ integrin is required for effective tyrosine phosphorylation of pp72$^{\text{syk}}$ and downstream phospholipase C_2 activation (15). Similarly, glycoprotein VI deficiency also results in the decreased phosphorylation of pp72$^{\text{syk}}$ during stimulation of platelets by collagen, despite normal expression of $\alpha_2\beta_1$ integrin (21). Nevertheless, the activation of platelets by collagen involves rapid phosphorylation of pp72$^{\text{syk}}$ independently of Ca$^{2+}$ fluxes, ADP release and platelet aggregation (5, 17). The platelet FcγRII also becomes tyrosine phosphorylated by collagen in a reaction which is upstream of pp72$^{\text{syk}}$ activation and independent of $\alpha_2\beta_1$ integrin (15). However, the precise role of this reaction in collagen-induced signaling in platelets is not clear.

Platelet aggregation can be inhibited by snake venom components which mainly belong either to a well known group of RGD-containing polypeptides (disintegrins) or to the group of hemorrhagic metalloproteinases. The RGD sequence in the disintegrins is recognized by $\alpha_1\beta_1$ integrin and their binding to platelets effectively inhibits adhesive protein-mediated cell-cell interaction (27, 28). This results in inhibition of platelet aggregation by agonists such as thrombin or collagen without interfering with either ATP/ADP release or rise in intracellular calcium (29, 30). These inhibitors, therefore, do not appear to influence intracellular events during platelet/agonist interac-

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§ To whom correspondence should be addressed: Dept. of Haematology, Royal Liverpool Hospital, Prescot Street, Liverpool L69 3BX, UK. Tel.: 44-151-706 4311; Fax: 44-151-706 4311/5810.
tion but rather interfere with the ligand binding to activated RGD-binding integrins. In contrast, venom metalloproteinases inhibit platelet function (31, 32) by a mechanism which is less well defined.

We have investigated platelet interaction with the 52-kDa hemorrhagic enzyme from *Bothrops jararaca* venom, jararhagin, which belongs to the class of venom metalloproteinases possessing a disintegrin-like domain (33). This domain has a high degree of homology with the RGD-containing disintegrins but in jararhagin the RGD sequence is replaced by ECD sequences (33, 34). It has been proposed that the disintegrin domain of jararhagin binds to the $a_2$ subunit I domain of $\alpha_\beta_3$ integrin (35, 36). The proposal that jararhagin recognizes $\alpha_\beta_3$ integrin is supported by our studies, which show that this enzyme specifically inhibits the interaction of platelets with collagen (37) and cleaves the $\beta_3$ subunit of this main platelet collagen receptor (36). Moreover, we have shown that jararhagin selectively inhibits the secretion-dependent phase of collagen-induced platelet aggregation and the ability of collagen to induce serine/threonine phosphorylation of pleckstrin, indicating that the enzyme impairs signal transduction leading to protein kinase C activation (38). Since in jararhagin-treated platelets $\alpha_\beta_3$ integrin is functionally intact (36, 39), the combined use of jararhagin and RGDC-containing disintegrins provides an opportunity to assess the relative contribution of $\alpha_\beta_3$ and $\alpha_{IIb} \beta_3$ integrins to collagen-induced platelet signaling.

Our results show that although both types of venom component inhibit platelet aggregation, only jararhagin abolishes collagen-induced phosphorylation of pp72$^{\text{tyk}}$ kinase. In contrast, the RGDC-containing disintegrins do not interfere with pp72$^{\text{tyk}}$ activation by collagen, but inhibit platelet aggregation due to their occupancy of $\alpha_{IIb} \beta_3$ integrin. Our findings that ADP-activated jararhagin-treated platelets signal for pp72$^{\text{tyk}}$ phosphorylation and aggregate with collagen and that this can be inhibited by the RGDS peptide, show that signaling by collagen requires both $\alpha_\beta_3$ and $\alpha_{IIb} \beta_3$ integrins. Since jararhagin cleaves the $\beta_3$ subunit of $\alpha_\beta_3$ integrin, we propose that this subunit is involved in the collagen-induced signaling resulting in pp72$^{\text{tyk}}$ activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Jararhagin (52 kDa) was purified from the venom of *B. jararaca* (33) and contortrostatin (13.5 kDa) from the venom of *Aghis troton contortorx contortrix* (40). Collagen was obtained from Horm Chemie (Munich, Germany) and human fibrinogen from Kabi Diagnostica (Stockholm, Sweden). Antibodies of following specificities were used: pp72$^{\text{tyk}}$, pp125$^{\text{AK}}$, phosphotyrosine (PY20-HRP) (Santa Cruz Biotechnology, Santa Cruz, CA); Fc$\gamma$RII (IV.3 mAb from Medarex, NJ); rabbit and mouse IgG-HRP conjugates (Transduction Laboratories, Lexington, KY); $\beta_3$ subunit (B44 mAb from Serotec, Oxford, United Kingdom, and a polyclonal antibody from Chemicon International, Temecula, CA); phosphoserine and phosphothreonine (Zymed, San Francisco, CA). Echistatin, RGDS peptide, enolase, lactoperoxidase, ATP; ADP, thrombin, prostaglandin E$_1$; leupeptin, pepstatin, 1,10-phenanthroline, bovine serum albumin, and molecular weight markers were purchased from Sigma (Dorset, UK); sodium $[^{32}]$H-thiophosphate, sodium $[^{35}]$sulfate (Na$[^{35}]$S), ascorbate, $[^{14}]$C-proline, $[^{32}]$P-ATP, enhanced cholinuminescence (ECL) reagent, and Hyperfilm$^{TM}$ from Amer sham Corp. (Buckinghamshire, UK); Immobilon-P membrane from Millipore (Bedford, MA); dithiobis(succinimidylpropionate) from Pierce; Sepharose 2B from Pharmacia Biotechnology (Up psala, Sweden); protein G-Sepharose from Zymed, and aprotinin from Bayer (Newbury, UK). All other chemicals used were of analytical grade.

**Platelet Isolation and Radiolabeling**—Venous blood from healthy donors was mixed with 3.8% trisodium citrate (9:1 v/v) and centrifuged at 125 × g for 10 min at room temperature. The supernatant platelet-rich plasma was separated and further centrifuged for 30 min at 1,500 × g on a 25/34% albumin gradient at room temperature. Platelets were collected between the two albumin layers and gel-filtered on a Sepharose 2B column equilibrated with Tyrode’s Heps buffer, pH 7.4 (138 mM NaCl, 3 mM KCl, 1 mM MgCl$_2$, 1 mM glucose, 0.5 mM NaH$_2$PO$_4$, 20 mM Hepes) containing 0.5% bovine serum albumin (41). For $[^{32}]$P-labeling, a platelet suspension (3 ml), prepared as described above except that phosphatase was omitted from the buffer, was incubated with 500 $\mu$Ci of $[^{32}]$P at 37 °C for 60 min. $[^{32}]$P-Labelled platelets were separated from unbound label by gel filtration as described above. For $[^{31}]$P-labeling, platelets in platelet-rich plasma were treated with 1 $\mu$g/ml prostaglandin E$_1$ and then washed in albumin-free Tyrode’s Heps buffer prior to iodination by the lactoperoxidase-catalyzed reaction as described above.

**Platelet Activation**—Platelet aggregation ($3 \times 10^9$ cells/ml) was initiated by adding either 2 $\mu$g/ml collagen, 0.05 unit/ml thrombin, 2 $\mu$M ADP, or 10 $\mu$g/ml IV.3 mAb subsequently cross-linked with 50 $\mu$g/ml anti-mouse Ig1g. Platelet aggregation by ADP was carried out in the presence of 200 $\mu$g/ml fibrinogen. Platelet aggregation by collagen does not require external fibrinogen because it is mediated by the binding of secreted agranular protein C9 which serves as substrate for ADP-activated platelet caspase.

**Platelet Protein Phosphorylation and Western Blotting**—For analysis of phosphorylated proteins in serine/threonine residues, unstimulated or ADP-stimulated ($[^{32}]$P-labeled) platelets ($3 \times 10^9$ cells/ml) were lysed with 100 mM Tris, pH 7.5, containing equal volumes of cold 2 × radioimmuno precipitation assay (RIPA) buffer 1 (25 mM Tris-HCl, pH 7.4, buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 $\mu$M sodium vanadate, 1 mM phenylmthanesulfonyl fluoride, and 1 $\mu$g/ml each of pepstatin and leupeptin) for 30 min at 4 °C. After centrifugation at 13,000 × $g$ for 30 min at 4 °C, the lysates (30 $\mu$l) were mixed with equal volumes of double strength sample buffer containing SDS and 4% 2-mercaptoethanol and electrophoresed in 5–15% polyacrylamide gels (42). Dried gels were autoradiographed. For analysis of protein phosphorylated on tyrosine, unstimulated or stimulated unlabeled platelets were lysed with an equal volume of cold 2 × RIPA buffer 2 (158 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, in 10 mM Tris-HCl, pH 7.2, containing 1 mM sodium vanadate, 1 mM phenylmethanesulfonyl fluoride and 100 $\mu$g/ml of protein C9) for 15 min at 4 °C. The insoluble residues were removed by centrifugation at 13,000 × $g$ for 15 min at 4 °C. Lysates (37 $\mu$l) were mixed with the same volume of sample buffer, boiled for 3 min and electrophoresed on a 7.5% polyacrylamide gel under reducing conditions. After electrophoresis, the proteins were transferred for Western blotting to Immobilon-P membranes. Membranes were blocked with 5% albumin in blocking buffer (1% BSA (Sigma), 0.1% sodium azide, 1% Triton X-100, Tris-HCl pH 7.5, containing 0.12% Nonidet-P-40) and incubated with PY20-HRP in blocking buffer containing 3% albumin for 1 h. Membranes were washed 4 times in blocking buffer; protein blots were developed with ECL reagent and then immediately exposed to film.

**Immunoprecipitation**—Platelet lysates (up to 10$^9$ platelets/ml) were precleared with protein G-Sepharose for 1 h, antibody (1 $\mu$g) was added, and the mixtures were incubated overnight at 4 °C. The immune complexes were subsequently precipitated with protein G-Sepharose for 1 h at 4 °C. Beads were washed four times in 1 × RIPA buffer 2, and the samples were electrophoresed in 7.5% polyacrylamide gels. After electrophoresis of immunoprecipitates, proteins were analyzed by Western blotting as described above. For analysis of immunoprecipitated protein, PY20 immunoblots were treated for 30 min at 50 °C with stripping buffer (62.5 mM Tris-HCl, pH 6.7, containing 2% SDS and 100 mM 2-mercaptoethanol). Membranes were then washed twice with large volumes of blocking buffer, blocked with 5% albumin in blocking buffer, and probed with an appropriate primary antibody for 1 h. They were then washed four times and probed with a secondary HRP-conjugated antibody for 30 min. Membranes were finally washed, and blots were developed with ECL reagent as above.

**Chemical Cross-linking of Platelet Proteins**—Unstimulated or collagen-stimulated platelets (10$^9$ cells/ml) were incubated with 500 $\mu$M of the membrane-permeable cross-linker, diethiodi(succinimidylpropionate), for 25 min at room temperature. The reaction was quenched by adding 50 mM Tris-HCl, pH 7.5. After 15 min, cells were centrifuged, the pellet resuspended in Tyrode’s Heps buffer and finally lysed with cold 2 × RIPA buffer 2 as above. Proteins were immunoprecipitated from the lysates by appropriate antibodies as described above. Immunoprecipitates were either analyzed under reducing conditions by SDS-
RESULTS

Jararhagin and RGD-containing Disintegrins Inhibit Platelet Aggregation by Distinct Mechanisms—We have confirmed that jararhagin inhibits platelet aggregation by collagen and have also demonstrated that contortrostatin, an RGD-containing disintegrin, inhibits this platelet response. In contrast to the inhibition caused by jararhagin, the inhibition of the platelet response to collagen due to contortrostatin was never complete (Fig. 1).

α1β3 integrin, the major platelet receptor found in an inactive conformational state in unstimulated platelets, requires activation to bind ligands (43). Platelet agonists via their specific receptors transduce a cascade of signals ultimately leading to the activation of α1β3 integrin to a high affinity state capable of binding soluble ligands (44). To verify whether α1β3 integrin can be activated in either jararhagin- or contortrostatin-treated platelets, we stimulated these platelets with ADP.

The addition of suboptimal amounts of ADP (0.2 μM) restored the aggregation of jararhagin-treated platelets by collagen; this was inhibited by the RGDS tetrapeptide (Fig. 1) known to block ligand-α1β3 binding. This peptide also inhibited the collagen-induced aggregation of normal platelets (not shown). Similar ADP stimulation of contortrostatin-treated platelets did not restore any response to collagen (Fig. 1).

Fig. 2 shows that ADP-induced aggregation of jararhagin-treated platelets in the presence of fibrinogen was similar to that of control platelets, demonstrating binding of fibrinogen to the activated α1β3 integrin. As expected, contortrostatin inhibited this reaction. Platelets treated with jararhagin also aggregated upon stimulation by thrombin (38), whereas those treated with contortrostatin, echistatin, or RGDS peptide did not (data not shown). It thus appears that jararhagin and RGD-containing disintegrins use different mechanisms to inhibit platelet responses.

The RGD-containing venom disintegrins target α1β3 integrin and, through the occupancy of this receptor, they inhibit platelet aggregation. In contrast, our findings that platelet aggregation by either thrombin, ADP alone, or ADP plus collagen is preserved in jararhagin-treated platelets clearly indicate that jararhagin does not target the α1β3 integrin. The fact that platelet aggregation by collagen alone is inhibited by jararhagin points to the specificity of this enzyme for α1β3 integrin whereby jararhagin degrades the β3 subunit of this collagen receptor as shown in Fig. 3. The data in Fig. 3 were obtained using 14C-mAb which in these experiments precipitated the β3 subunit only, without associated α3 or α5 subunits as seen with other anti-β3 mAbs (36). The 130-kDa band shown in this figure therefore contains only β3 protein without co-precipitated 135-kDa component of the reduced α3 chain. Fig. 3 also shows that part of β3 protein was not degraded by jararhagin. This can be explained by preservation of the fraction of β3 associated with α5, in contrast to α3, which lacks the I domain required for jararhagin binding to the integrin heterodimer. We next investigated the serine/threonine and tyrosine phosphorylations of some proteins involved in platelet activation to gain further insight into the mechanisms of inhibition of platelet stimulation with collagen by these venom components.

Phosphorylation of Pleckstrin (p47) Indicates Different Effects of Contortrostatin and Jararhagin on Platelet Stimulation Signaling—Following agonist stimulation, serine/threonine phosphorylation of pleckstrin (45) is associated with platelet granule secretion (46, 47). We have already shown that in platelets treated by jararhagin, the secretion-associated p47 phosphorylation in response to collagen is diminished (38); Fig. 4 (i) shows the results of p47 phosphorylation, confirming that
this platelet response to collagen is inhibited by jararhagin (lane 3). However, p47 phosphorylation was preserved when 32P-labeled jararhagin-treated platelets were preactivated by ADP before exposure to collagen, although ADP alone (0.2 μM) did not induce this phosphorylation. In contrast, in contortrostatin-treated platelets p47 was normally phosphorylated in response to collagen in the absence or presence of ADP. The phosphorylation of p47 was fully preserved in both jararhagin- and contortrostatin-treated platelets stimulated with thrombin (Fig. 4 (ii)). These results thus show that the secretion-inducing pathway, when activated by collagen alone, was inhibited in jararhagin- but not in contortrostatin-treated platelets. The results of p47 phosphorylation in contortrostatin-treated platelets concur with preserved granule secretion recorded in platelets treated with other RGD-containing disintegrins (29, 30).

Platelet Protein Tyrosine Phosphorylation in Response to Collagen Is Inhibited by Jararhagin but Not by RGD Disintegrins—Fig. 5 (i) shows immunoblots of tyrosine-phosphorylated proteins from platelet lysates. Stimulation of untreated platelets with collagen caused increased phosphorylation of proteins in the 50–76-kDa and 125-kDa ranges. Jararhagin-treated platelets, either nonstimulated or stimulated by collagen, did not show an increase in protein phosphorylation compared with untreated controls. However, when jararhagin-treated platelets were exposed to 0.2 μM ADP, protein tyrosine phosphorylation clearly increased in response to platelet stimulation by collagen. These results demonstrate that jararhagin itself does not stimulate platelets and fully support the restoration of the platelet aggregation response to collagen with suboptimal ADP treatment (Fig. 1, trace 4).

Similar experiments were carried out with platelets treated...
with contortrostatin and echistatin. Results showed that, despite strong reduction of aggregation, protein tyrosine phosphorylation was fully preserved in contortrostatin-treated platelets when induced with either collagen alone or collagen and ADP (Fig. 5 (i)). However, the phosphorylation of a band of about 125 kDa (indicated by an arrowhead), which was preserved in contortrostatin-treated cells (Fig. 5 (i) lanes 7 and 8), was absent in echistatin-treated platelets. The 125-kDa band was identified as the fucosyl adhesion kinase, pp125^FAK^, by Western blotting (not shown).

To determine the most prominent substrates of tyrosine kinase(s) responding to collagen stimulation, 32P-labeled platelets were also used. When these were treated with native jararhagin and then stimulated with collagen, analysis of PY20-immunoprecipitated proteins showed reduced protein tyrosine phosphorylation compared with the control. In particular, the absence of the major doublet labeled protein in the region of 70 kDa and strong reduction in phosphorylation of a 125-kDa protein (pp125^FAK^) were recorded (Fig. 6 (i)). Such a reduction in protein tyrosine phosphorylation in response to collagen is not observed in glycoprotein VI-deficient platelets expressing normal α1β1 integrin (21) further confirming that the effect of jararhagin is restricted to α1β1 signaling. We next examined the phosphorylation state of pp72^yk_1 in platelets. The result of these experiments is shown in lane 4 (Fig. 6 (ii)).

The Phosphorylation of Tyrosine Kinase pp72^yk_1 in Collagen-stimulated Platelets Is Decreased by Jararhagin—In control platelets stimulated with collagen, pp72^yk_1 was clearly phosphorylated (Figs. 4 (ii) and 6 (ii), lanes 2). However, when platelets were pretreated with jararhagin this response to collagen was markedly decreased. Thus, pp72^yk_1 was not phosphorylated in jararhagin-treated platelets and this resembles the earlier observations in glycoprotein VI-deficient platelets by Ichinobe et al. (21). Platelets treated with RGD-disintegrins had phosphorylated pp72^yk_1 despite the inhibition of aggregation when induced with either collagen alone or in association with ADP (Fig. 4 (ii) and 5 (ii)). Also, jararhagin, contortrostatin, or echistatin alone did not cause an increase in pp72^yk_1 phosphorylation. The suboptimal amounts of ADP alone used in these studies did not cause substantial pp72^yk_1 phosphorylation (Fig. 4 (ii)). This implies that although the α1β3 integrin had been activated, pp72^yk_1 is phosphorylated only when collagen binds to this integrin. In platelets stimulated by thrombin, pp72^yk_1 phosphorylation was not affected by either jararhagin or contortrostatin (Fig. 4 (ii)). The respective total immunoprecipitated pp72^yk_1 blots are shown under the blots of phosphorylated pp72^yk_1 (Fig. 5 (iii)).

These results in our experimental model demonstrate that the ability to cause pronounced inhibition of collagen-induced pp72^yk_1 phosphorylation is a specific property of jararhagin. Since we have previously demonstrated (36), and confirmed here, that jararhagin cleaves the platelet α1β1 integrin, this proteolysis could be the most likely cause of the inhibition of platelet interaction with collagen leading to pp72^yk_1 phosphorylation.

Platelet β1 Subunit of α1β1 Is Not Directly Associated with a Protein Kinase—Jararhagin cleaves the β1 subunit of α1β1 integrin (Fig. 3) and, in other cell types, β1 has been shown to associate with cytoskeletal proteins (48, 49) and to play an important role in signaling events (50, 51). Therefore, we next searched for possible kinase activity associated with the β1 subunit, although attempts to demonstrate this have failed in the past (26). However, since in the latter report (26) immunoprecipitates were prepared with an anti-β1 antibody, we carried out similar experiments using anti-β1 antibody and employed a membrane-permeable chemical cross-linker in the search of other proteins that may be associated with the cytoplasmic tail of the immunoprecipitated protein. Although we examined either non-cross-linked or chemically cross-linked resting and collagen-activated platelets, we could detect neither a phosphoprotein nor protein-tyrosine kinase activity in these immunoprecipitates (not shown). These results therefore confirm the absence of protein kinase association with the α1β1 integrin in platelets as previously reported by Asazuma et al. (26).

DISCUSSION

Here we have demonstrated that the venom metalloproteinase jararhagin inhibits an early stage of signaling in collagen-stimulated platelets, markedly reducing the phosphorylation of the tyrosine kinase pp72^yk_1. The defective signaling in jararhagin-treated platelets can be attributed to the previously demonstrated proteolysis of α1β1 integrin by jararhagin, clearly indicating the involvement of this integrin in signaling for pp72^yk_1 phosphorylation by collagen. The activation of platelets by ADP restores the ability of jararhagin-treated platelets to generate this signal most likely because collagen binds to the activated α1β3 integrin (10). In contrast, two RGD-containing venom disintegrins, contortrostatin and echistatin, inhibited platelet aggregation by collagen but did not impair collagen-induced phosphorylation of pp72^yk_1. It is therefore likely that the occupancy of the major platelet adhesion/aggregation receptor α1β3 integrin by these disintegrins, without any interference with signaling by α1β1 integrin, is the main cause of inhibition of platelet aggregation in response to collagen. Thus, the present investigation shows that two distinct classes of venom-derived components, metalloproteinases and RGD-containing disintegrins, inhibit platelet function differently.

Jararhagin selectively inhibits collagen-induced platelet responses by first recognizing the I domain of α1β1 integrin (35) followed by proteolysis of the β1 subunit of the integrin (36). Both of these events are probably important for the resulting alterations in the functional properties of this integrin. Thus, we have found that upon inactivation of its
catalytic site, jararhagin still binds to platelets (36) most probably via the part of its disintegrin domain containing the SEC6 sequence (38). Since we could not demonstrate an equilibrium binding of active jararhagin to platelets, it is likely that the proteolysis of the β3 chain affects the binding properties of the receptor for both the enzyme and collagen.

The demonstration that jararhagin-treated platelets respond to collagen in the presence of ADP suggests that platelet αIIbβ3 integrin and collagen retain their mutual binding capacities. ADP activates platelet αIIbβ3 integrin to bind RGD-containing ligands (43, 44), including collagen (10), and this binding is presumably responsible for the restored signal transduction observed here. Since it is known that platelet stimulation with agonists such as thrombin increases the expression of platelet αIIbβ3 integrin by about 20% (52), it is possible that our observation of restored collagen-induced platelet aggregation in jararhagin-treated platelets in the presence of ADP might have been the result of such an increase. However, this seems unlikely because this newly expressed αIIbβ3 was also available for the attack by jararhagin during the incubation preceding platelet stimulation by collagen. Moreover, our demonstration that the restoration of platelet response to collagen by ADP is greatly reduced by the RGDS peptide, further supports our proposal that in ADP-stimulated jararhagin-treated platelets collagen binds to the activated αIIbβ3 integrin.

In contrast to jararhagin, the RGD-containing venom disintegrins impaired the platelet aggregation with either collagen alone or collagen and ADP, but did not interfere with pp72"syk" activation. Since these disintegrins block αIIbβ3 integrin but do not interfere with the interaction of collagen with αIIbβ3 integrin, the latter interaction is presumably responsible for the preserved pp72"syk" phosphorylation observed in the presence of these agents. We also found that contortrostatin did not inhibit platelet pp72"syk" activation in response to stimulation by thrombin as previously reported by Clark et al. (53). It has been reported that contortrostatin alone can trigger pp72"syk" activation but at much higher concentrations (>500 nM) (53) than were used in the present study (100 nM). This effect, attributed to the dimeric structure of contortrostatin was not seen in our studies but, at equimolar concentrations, contortrostatin was a less effective platelet inhibitor than the monomeric disintegrin echistatin as judged by photophosphorylation blots shown in Fig. 5. In platelets treated with the RGD-containing disintegrins, collagen-induced pp72"syk" phosphorylation is followed by a series of downstream events including protein kinase C activation and phosphorylation of pleckstrin (Fig. 4), as well as granule secretion (29, 30). In contrast, we have shown that in jararhagin-treated platelets not only aggregation but also pleckstrin phosphorylation and granule secretion in response to collagen are absent. It appears therefore that all signals, with the exception of that responsible for myosin light chain phosphorylation and platelet shape change (38), are inhibited by the treatment of platelets with jararhagin.

Studies of integrin signaling in platelets have been mostly concerned with the αIIbβ3 integrin. It has been shown that the cytoplasmic tail of the β3 subunit is vital for the initiation of the outside-in signaling by this integrin (54, 55) and that the β3 subunit coprecipitates with Src family kinases (55, 56). Recent studies with other cells have demonstrated that the β1 subunit of β3 integrins also participates in signaling. During adhesion of epithelial cells to fibronectin, the β1 subunit of the αIIbβ3 integrin associates with the 59-kDa integrin-linked phosphoprotein, p59DQK, providing a downstream link with other cytoplasmic and cytoskeletal proteins (57). Upon fibroblast adhesion to the extracellular matrix, the β1 subunit of the αIIbβ3 integrin also coaggregates with pp125FAK (58) and paxillin (59). Moreover, expression of various modified transmembrane or cytoplasmic domains of the β1 subunit results in decreased activation of protein tyrosine phosphorylation in transformed cells (51). All this evidence, therefore, supports our proposal that the αIIbβ3-dependent signal for activation of protein tyrosine kinases in platelets stimulated by collagen requires intact β3 subunit of the αIIbβ3 integrin. However, since association of kinase activity with this subunit could not be demonstrated either in our studies or in those of others (26), the nature of link between αIIbβ3 and the protein tyrosine kinase cascade in platelets remains to be established.

Although the αIIbβ3 integrin seems to be relevant for the platelet response to collagen both in our investigations and in those of others (7, 9, 15, 25, 26), evidence for the role of glycoprotein VI in collagen-induced platelet signaling cannot be ignored (12, 21, 23). The findings that platelet pp72"syk" cannot be phosphorylated in the absence of either αIIbβ3 alone or glycoprotein VI alone, led to the suggestion that cooperation between these two receptors is necessary for collagen-induced signaling (21). Our preliminary experiments using chemical cross-linking have so far failed to demonstrate physical association of αIIbβ3 integrin and glycoprotein VI in normal platelets (not shown). Also we could not investigate whether jararhagin has any direct effect on glycoprotein VI in the present study because we could not obtain an antibody against this glycoprotein.

Apart from pp72"syk", FcγRII is also tyrosine phosphorylated at an early stage during the platelet-collagen interaction (15). It is known that binding of IV.3 mAb to FcγRII induces platelet aggregation (59–61) and that this is accompanied by the phosphorylation of the receptor itself, pp72"syk" and pp125FAK (3, 62), but not of the FcγRII receptor. Therefore, the absence of pp72"syk" phosphorylation observed with jararhagin-treated platelets cannot be attributed to a change in FcγRII. This further reinforces our proposal that this defect in platelet signaling is caused by the action of jararhagin on the αIIbβ3 integrin.

Platelet glycoprotein IV (CD36) is also implicated as a primary receptor for platelet adhesion to collagen (11) and, when stimulated by antibodies, generates the signal for platelet aggregation (63). However, platelets lacking glycoprotein IV can be fully aggregated with collagen and show normal protein tyrosine phosphorylation (19, 64). Our unpublished observation that a monoclonal antibody against glycoprotein IV (OKM5) promotes full aggregation of jararhagin-treated platelets, excludes a possible effect of jararhagin on this receptor.

In conclusion, our results demonstrate impaired signaling by collagen in platelets following proteolysis of the αIIbβ3 integrin by jararhagin. In particular, this is the first report of the loss of pp125FAK (58) and paxillin (59). Moreover, expression of various modified transmembrane or cytoplasmic domains of the β3 subunit results in decreased activation of protein tyrosine phosphorylation in transformed cells (51). All this evidence, therefore, supports our proposal that the αIIbβ3-dependent signal for activation of protein tyrosine kinases in platelets stimulated by collagen requires intact β3 subunit of the αIIbβ3 integrin. However, since association of kinase activity with this subunit could not be demonstrated either in our studies or in those of others (26), the nature of link between αIIbβ3 and the protein tyrosine kinase cascade in platelets remains to be established.

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Apart from pp72"syk", FcγRII is also tyrosine phosphorylated at an early stage during the platelet-collagen interaction (15). It is known that binding of IV.3 mAb to FcγRII induces platelet aggregation (59–61) and that this is accompanied by the phosphorylation of the receptor itself, pp72"syk" and pp125FAK (3, 62), but not of the FcγRII receptor. Therefore, the absence of pp72"syk" phosphorylation observed with jararhagin-treated platelets cannot be attributed to a change in FcγRII. This further reinforces our proposal that this defect in platelet signaling is caused by the action of jararhagin on the αIIbβ3 integrin.

Platelet glycoprotein IV (CD36) is also implicated as a primary receptor for platelet adhesion to collagen (11) and, when stimulated by antibodies, generates the signal for platelet aggregation (63). However, platelets lacking glycoprotein IV can be fully aggregated with collagen and show normal protein tyrosine phosphorylation (19, 64). Our unpublished observation that a monoclonal antibody against glycoprotein IV (OKM5) promotes full aggregation of jararhagin-treated platelets, excludes a possible effect of jararhagin on this receptor.

In conclusion, our results demonstrate impaired signaling by collagen in platelets following proteolysis of the αIIbβ3 integrin by jararhagin. In particular, this is the first report of the loss of signal for tyrosine phosphorylation of pp72"syk" attributable to an acquired defect in the function of αIIbβ3 integrin. Moreover, the signaling was largely restored by ADP stimulation apparently through collagen binding to activated αIIbβ3. Thus, although the precise pathway of collagen-induced signaling in platelets still remains unresolved, our findings draw attention to the importance of both αIIbβ3 and αIIbβ3 integrins in this process.

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