Integrin-independent Tyrosine Phosphorylation of p125fak in Human Platelets Stimulated by Collagen*

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Collagen fibers or a glycoprotein VI-specific collagen-related peptide (CRP-XL) stimulated tyrosine phosphorylation of the focal adhesion kinase, p125fak (FAK), in human platelets. An integrin αIIbβ3-specific triple-helical peptide ligand, containing the sequence GFOGER (single-letter nomenclature, O = Hyp) was without effect. Antibodies to the α2 and β1 integrin subunits did not inhibit platelet FAK tyrosine phosphorylation caused by either collagen fibers or CRP-XL. Tyrosine phosphorylation of FAK caused by CRP-XL or thrombin, but not that caused by collagen fibers, was partially inhibited by GR144053F, an antagonist of integrin αIIbβ3, which supports both platelet adhesion and activation (5, 6). At present, α2β1 is considered primarily an adhesive co-receptor (7), whereas other collagen receptors, notably glycoprotein VI, activate platelets (8). This study was designed to clarify which collagen receptors transmit signals to the platelet interior, information which is crucial for the development of anti-platelet therapy based on collagen receptor antagonism (9).

Recent evidence suggests that CD36, α2β1, and GPVI each contribute to both signaling and adhesion to collagen (10). GPVI, recently cloned (11), acts with the Fc receptor γ-chain (12, 13) as a crucial signaling receptor complex, and platelets deficient in GPVI fail to aggregate in response to collagen, although the tyrosine kinase c-Src, but not p125fak, is activated (14). The role of α2β1 in platelet signaling is unclear: α2β1-reactive snake venoms fuel the debate on the integrin’s role in platelet signaling (15, 16), and overexpression of α2β1 has recently been advanced as a risk factor in myocardial infarction and stroke (17, 18).

We have synthesized a collagen-related peptide (CRP) recognized by GPVI, which shares both the triple-helical structure and activatory characteristics of collagen (19). CRP comprises a repeating GPO motif, a sequence representing about 10% of the primary structure of collagen. CRP, when cross-linked (CRP-XL), is a potent platelet agonist depending only upon GPVI for its activity. Several lines of evidence show that CRP-XL is not recognized by α2β1 (19–24).

Recently, we have developed a triple-helical peptide containing the sequence GFOGER, which is a high affinity binding motif for the α2 I domain (25). This peptide, GP-[GPP]$_5$-GFOGER-[GPP]$_{15}$-GPC, designated GFOGER-GPP, supports platelet adhesion, as well as collagen, but does not activate platelets in suspension even when cross-linked (GFOGER-GPP-XL), nor does it stimulate obvious tyrosine phosphorylation (23). Its binding to platelets is fully abrogated by the α2-specific monoclonal antibody 6F1. The sequence GFOGER has been co-crystallized with the α2 I domain, demonstrating its binding to the metal ion-dependent adhesion site (26).

CRP-XL and collagen elicit very similar signals from platelets, activating protein kinase C (PKC) (27), mobilizing arachidonic acid from platelet membranes (19), and Ca$^{2+}$ from intracellular stores (27); CRP-XL activates p38 mitogen-activated protein kinase (28) and p72syk and leads to tyrosine phosphorylation of many platelet proteins, including phospholipase Cγ2 (29) and the Fc receptor γ-chain (12). CRP-XL, like collagen, activates platelet procoagulant expression (24). These studies emphasize the importance of GPVI as a collagen receptor in platelets.

Platelet activation leads to the up-regulation of tyrosine kinases, including FAK (30), p72syk (31), and members of the c-Src family (32). FAK, a 125-kDa cytosolic non-receptor tyrosine kinase, is associated with focal adhesion plaques of adher-
en cells such as fibroblasts and platelets (33). FAK is of particular interest, because it is considered a key intermediary of signaling through integrins (34–36).

Phosphorylation of FAK occurs at five tyrosine residues and correlates with an increase in FAK tyrosine kinase activity. Autophosphorylation of tyrosine 397 allows it to bind the c-Src and PKC in platelet FAK activation using the Ca\(^{2+}\)-dependent protease calpain, which leads to a reduction in its autophosphorylation (41).

Evidence from fibroblasts suggests that occupation of β1 integrins is a sufficient stimulus to activate FAK (35, 42, 43). Collagen binding to α\(_1\)β\(_1\) in T cells protects them from apoptosis in a FAK-dependent manner (36). Adhesion of platelets to monomeric collagen occurs through α\(_2\)β\(_1\). A causal relationship has been proposed between α\(_2\)β\(_1\) and FAK activation in platelets adherent to monomeric collagens (44–46). This does not prevent the movement of the two, site-specific anti-FAK antibodies, and determined the tyrosine phosphorylation of FAK as described previously (19, 25). Anti-phosphotyrosine 3-(2-pyridyldithio)propionic acid has been identified as a high-affinity ligand for human platelets (44–46). This does not correlate with an increase in FAK tyrosine kinase activity.

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Experimental Procedures

Materials—Collagen, as native type I fibers isolated from bovine tendons, was obtained from Ethicon, Inc., Somerville, NJ. CRP and GFOGER-GPP (Gly-Pro-Cys-Gly-Pro-Pro-Gly-Pro-Hyp-Gly-Glu-Arg-Gly-Pro-Pro-Gly-Pro-Cys) were synthesized and cross-linked using 3-(2-pyridyldithio)propionic acid and N-hydroxysuccinimide ester (P-3415, Sigma, UK) as described previously (19, 25). Anti-phosphotyrosine (clone 4G10) was from Upstate Biotechnology Inc., Lake Placid, NY. Anti-FAK (catalog no. F15020/L4) was from Affiniti Research Products, Nottingham, UK, and C-20 (catalog no. sc-558) was from Santa Cruz Biotechnology, CA. Anti-α\(_2\)β\(_1\) mAbs, 6F1 and P1E6, were a kind gift from Dr. B. S. Coller, School of Medicine, State University of New York, Stony Brook, NY, and obtained from Calbiochem-Novabiochem (UK) Ltd., Nottingham, UK, respectively. The anti-β1 mAbs, 2A4 and mAb13, were from Genosys Biotechnologies, Cambridge, UK, and from Becton Dickinson, Oxford, UK, respectively. Horseradish peroxidase-linked anti-mouse whole antibody from sheep (NA931), Rainbow molecular weight markers (RPN756), [\(^{32}\)P]orthophosphate, and Hybrid C N-acrolein (RPN303C) were from Amersham Pharmacia Biotech, UK. Thrombin (T-2425), apyrase (A-6535), aspirin (A-5376), 12-O-tetradecanoylphorbol-13-acetate (TPA, T-2103), bovine albumin fraction V (A-4503), Pansorbin (P-2884), phenylmethylsulfonylfuoride (P-7626), benzamidine (B-6506), and luminol (A-6685) were all from Sigma, UK. Chemicals for electrophoresis were Electran grade from BDH Laboratory Supplies, Poole, UK. 4-Iodofenol (I1, 020–1) was from Aldrich Chemical Co., Gillingham, UK. H\(_2\)O\(_2\) (H1800/07) was from Fisons Scientific Equipment, Loughborough, UK, and RK medical x-ray film was from Fuji Film Co., Ltd., Japan. X-ray developer (LX24) and fixer (FX540) were from Kodak Scientific, Cambridge, UK. BAPTA-AM (1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, tetraacetoxy-methyl ester; 196419), Pansorbin (507858), and Ro31-8220 (557520) were from Calbiochem-Novabiochem, Nottingham, UK, and Fura2-AM was from Molecular Probes, Eugene, OR. The fibrinogen receptor antagonist and RGD peptide mimetic, OGR44055F, was a generous gift from GlaxoWellcome, Stevenage, UK. All other chemicals were of standard reagent grade.

Platelet Preparation—Platelets concentrates, less than 24 h-old, pooled from four donors, were obtained from the National Blood Service, Long Road, Cambridgeshire, UK, centrifuged at 250 × g for 15 min to remove red blood cells, leaving platelet-rich plasma, from which the platelets were resuspended at 700 × 10\(^6\)/ml for 15 min. The platelet pellet was resuspended in loading buffer (LB; 145 mM NaCl, 5 mM KCl, 10 mM glucose, 1 mM MgSO\(_4\), 0.5 mM EGTA, 10 mM HEPES, pH 7.36). The platelets were pelleted at 700 × g for 10 min and resuspended in LB at 10\(^6\)/ml for immunoprecipitation and at 5 × 10\(^9\)/ml for other work. Aspirin (100 μM) and apyrase (0.25 units/ml) were used where indicated.

Immunoprecipitation—Platelet agonist suspensions (500 μl) were mixed with an equal volume of 2 × radioimmune precipitation buffer (2% Triton X-100, 2% sodium deoxycholate, 0.2% SDS (each w/v), 316 mM NaCl, 2 mM EGTA, 20 mM Tris/HCl, pH 7.2, with 10 mg/ml leupeptin, 10 μM benzamidine, 2 mM phenylmethylsulfonyl fluoride, and 2 mM Na\(_3\)VO\(_4\)), and incubated on ice for 30 min before centrifugation (13,000 × g) for 5 min at 4 °C. Pansorbin (60 μl/ml lystate) was added to each platelet lysate and the sample was rotated at 4 °C for 60 min. Blood samples were centrifuged (13,000 × g) for 1 min at 4 °C, resuspended in the original volume of 1 × radioimmune precipitation buffer, and allowed to stand at room temperature for 15 min. It was then centrifuged again and resuspended in 1× radioimmune precipitation buffer containing bovine serum albumin (1% w/v). Samples were centrifuged (13,000 × g) for 1 min at 4 °C, the supernatant was removed, and it to resuspended with 3× radioimmune precipitation buffer (3% Triton X-100, 3% sodium deoxycholate, 3% SDS, each w/v, 70 mM Tris/HCl, pH 7.2, with 1% 2-mercaptoethanol, w/v) and boiled for 5 min. Samples were divided in 2× 40 μl, and proteins were separated by 8% SDS-polyacrylamide gel electrophoresis then blotted to nitrocellulose (2 × 1 mAc/m\(^2\), Hoefer TE77 semi-dry blotter). Uniform protein transfer was verified by Poncso S staining. One blot was incubated with 4G10 (1:2500) and washed with TBST (20 mM Tris/HCl, 136 mM NaCl, 0.1% (w/v) Tween 20, pH 7.6), and anti-phosphotyrosine was detected using horseradish peroxidase-linked anti-mouse antibody (1:10,000) and enhanced chemiluminescence (1.24 mM luminol, 1.63 μM 4-iodophenol, 2.71 μM H\(_2\)O\(_2\)). Phosphorylation was quantitated densitometrically using a Leica Q500 image analyzer (51) and is expressed as a percentage change relative to control values. The other blot was probed with monoclonal anti-FAK (Affiniti, 1:1000), to verify uniform recovery of FAK. Each experiment was performed using different platelet preparation on three separate occasions.

Platelet Aggregation—Platelets were prepared as for immunoprecipitation and resuspended to 10\(^6\)/ml in LB. 150 μl of suspension was stirred (1100 rpm) in an aggregometer at 30 °C as described (19), and inhibitors or solvent were added and followed 5 min later by ligand in a volume corresponding to 2% final concentration.

To verify the inhibitory properties of the anti-β1 antibody, 2A4, washed platelets were prepared from whole blood (19), and preincubated in the aggregometer as above for 1 min with 2A4 (20 μg/ml), before addition of just sufficient collagen fibers to cause maximal aggregation.

Protein Kinase C Activity—Platelets were prepared as for immunoprecipitation and resuspended to 10\(^6\)/ml in LB. They were labeled with...
Collagen fibers at 25 different concentrations of collagen and CRP-XL. Equal recovery of FAK immunoprecipitated from platelets activated by concentration-dependent increase in the tyrosine phosphorylation of FAK was demonstrated in each sample. Affinity monoclonal anti-FAK for immunodetection. Again, precipitated FAK from CRP-XL-activated platelets, using the antibody otherwise as for a and b. Relative phosphorylation of FAK (Fig. 1). Fig. 1 shows equal recovery of FAK in each sample.

RESULTS

Immunoprecipitation of FAK—FAK, immunoprecipitated from platelets activated by collagen at 25 μg/ml, showed a time-dependent increase in tyrosine phosphorylation (Fig. 1a). The increase in tyrosine phosphorylation at 60 s was detectable but small; therefore, 5-min incubation, causing a substantial increase in FAK tyrosine phosphorylation, was chosen for subsequent assays. Fig. 1b shows equal recovery of FAK in each sample.

Immunoprecipitation of FAK from platelets activated by CRP-XL at 5 μg/ml showed a time-dependent increase in tyrosine phosphorylation of FAK (Fig. 1c). Fig. 1d shows immunoprecipitated FAK from CRP-XL-activated platelets, using the Affiniti monoclonal anti-FAK for immunodetection. Again, equal amounts of FAK were demonstrated in each sample.

Effect of Ligand Concentration—Fig. 2, a and d, shows a concentration-dependent increase in the tyrosine phosphorylation of FAK immunoprecipitated from platelets activated by different concentrations of collagen and CRP-XL. Equal recovery of FAK was demonstrated in all cases (data not shown). Collagen fibers at 25 μg/ml and CRP-XL at 5 μg/ml caused near-maximal increases in FAK tyrosine phosphorylation. Some experiments (data not shown) were performed in the presence of apyrase, which scavenges ADP secreted by activated platelets, and aspirin, which blocks the conversion of arachidonate to thromboxane A₂. The inhibitors had no marked effect, indicating that tyrosine phosphorylation of FAK does not depend upon these processes in platelets stimulated by collagen or CRP-XL.

In some experiments the basal level of FAK tyrosine phosphorylation was detectable, whereas others (e.g. Fig. 2e) showed negligible FAK tyrosine phosphorylation. This may reflect variation between donors, or in the activation state of resting platelets between experiments, as well as in the immunodetection procedure. Conclusions throughout this study are therefore based on comparisons made within an experiment, and where possible, within immunoblots rather than between blots.

Role of α₂β₁ in FAK Tyrosine Phosphorylation by Collagen and CRP-XL—When FAK was immunoprecipitated from platelets preincubated with anti-α₂ P1E6 or anti-β₁ 2A4 for 5 min...
before activation with collagen for 5 min, there was no diminu-
tion, confirmed by densitometry, in the level of tyrosine
phosphorylation of FAK induced by either ligand (Fig. 2b).
Similar data were obtained using the anti-β1 mAb13 (data not
shown) or the anti-α2, 6F1 (Fig. 5b).

CRP-XL induces platelet activation without involvement of
α,β1 and caused substantial tyrosine phosphorylation of FAK.
This shows that ligation of GpVI, the receptor for CRP-XL,
duces phosphorylation of FAK. As anticipated, the anti-α2
and anti-β1 antibodies had no effect on the tyrosine phospho-
rylation of FAK by CRP-XL.

Recent work in this laboratory has shown that the affinity of
platelet α,β1 is dependent upon the presence of micromolar Ca2+
in the suspending medium (54). For this reason, the experi-
ments shown above for collagen were repeated in the
presence of a small excess of Ca2+ over EGTA in the buffer,
conditions that support α,β1-dependent platelet adhesion to
immobilized collagens. FAK tyrosine phosphorylation was not
enhanced by the presence of Ca2+ compared with the parallel
incubation in the absence of Ca2+ (Fig. 2c).

We have recently shown the peptide sequence GFOGER to be
a recognition motif in type I collagen for the
α,β1 domain (25).

Application of the cross-linked triple-helical peptide, GFOGER-
GPP-XL, to platelets at up to 50 µg/ml caused no discernible
increase in FAK tyrosine phosphorylation (Fig. 3a). In contrast,
in this experiment as in Fig. 2a, collagen fibers caused sub-
stantial FAK tyrosine phosphorylation.

The addition of micromolar Ca2+ to the medium did not support FAK phosphoryla-
tion stimulated by even high levels of the peptide (200 µg/ml).

**Functional Verification of the Anti-α2,β1 Antibodies—6F1**
as used in the present study completely blocked platelet adhesion
to monomeric collagen (54, 55). Similar experiments showed
both P1E6 and mAb13 to be effective inhibitors of platelet
adhesion to monomeric collagen. The anti-α2, P1E6, blocked the
capacity of reconstituted type I collagen fibers to induce platelet
aggregation (7). We verified here that both P1E6 and the anti-β1, 2A4, could attenuate the platelet aggregation stim-
ulated by threshold concentrations of native collagen fibers
(data not shown). Together, these data confirm the functional
activity of the antibodies used here.

**Functional Verification of the α1β1 Antagonist GR144053F**—Fig.
4 shows that CRP-XL (5 µg/ml) or thrombin
(1 unit/ml), levels of agonist consistent with the rest of the
study, aggregated platelets suspended in medium containing
0.5 mM EGTA, but that collagen fibers (25 µg/ml) caused min-
imal platelet aggregation. Preincubation with the fibrinogen
receptor antagonist GR144053F (1 µM) reduced the extent of
aggregation to <15% of control values in platelets stimulated
by CRP-XL or thrombin.

**Effect of GR144053F on FAK Tyrosine Phosphorylation**—Fig.
5a shows that preincubation of platelets with 1 µM GR144053F,
a level which causes complete blockade of α1β1 (54), caused
a substantial reduction in FAK tyrosine phosphorylation in
platelets subsequently stimulated by CRP-XL (77% reduction
over four trials) or thrombin (63% over two trials). This effect
was of similar order to the inhibition (85%) of aggregation by
GR144053F for CRP-XL or thrombin. In contrast, there was
little observable inhibition (15%; five trials) of the action of
collagen by GR144053F, even when used in conjunction with
α2-blockade by 6F1 (Fig. 5b). Basal phosphorylation of FAK
was also inhibited to some extent (30%; four trials), perhaps
indicating a degree of activation of platelets under resting
conditions, consistent with the suggestion, above, that the ba-
sal platelet preparations might to some extent be activated.
This effect of GR144053F was minor compared with the
marked inhibition of the action of CRP-XL or thrombin.
Role of Protein Kinase C in FAK Tyrosine Phosphorylation—To investigate signaling pathways required for FAK tyrosine phosphorylation, we examined the role of protein kinase C. FAK was immunoprecipitated from platelets stimulated for 5 min with collagen (25 μg/ml), CRP-XL (5 μg/ml), or TPA (400 nM). As before, marked tyrosine phosphorylation of FAK was induced by collagen and CRP-XL, whereas control levels were undetectable (Fig. 6a). TPA caused a very minor increase in FAK tyrosine phosphorylation: densitometry showed that CRP-XL and collagen were each about 20 times more effective than TPA.

Effect of Ro31-8220 or BAPTA on Tyrosine Phosphorylation of FAK Stimulated by Collagen, CRP-XL, or Ionomycin—Fig. 6b shows complete inhibition of tyrosine phosphorylation of FAK immunoprecipitated from platelets after pretreatment with the PKC inhibitor Ro31-8220 (5 μM) prior to activation by collagen (25 μg/ml) or CRP-XL (5 μg/ml) for 5 min. We have shown 5 μM Ro31-8220 to cause complete inhibition of PKC, measured as p47 phosphorylation (56). The calcium ionophore, ionomycin, also caused substantial tyrosine phosphorylation of FAK (Fig. 6c), suggesting a role for calcium signaling in FAK activation, and again, as for collagen and CRP-XL, this action was substantially attenuated by Ro31-8220.

Preincubation of platelets with the Ca2+-chelating agent, BAPTA-AM, to buffer rises in intracellular Ca2+, markedly attenuated the ability of both CRP-XL and collagen fibers to stimulate tyrosine phosphorylation of FAK (Fig. 6d). For comparison, in Western blots prepared from whole platelet lysates there was an inhibition of overall tyrosine phosphorylation stimulated by collagen, CRP-XL and in the control samples of 12, 21, and 7%, respectively (Fig. 6e), when platelets were preincubated with BAPTA-AM. This inhibition indicated that the effects on FAK are highly specific. In contrast, one band of about 38 kDa increased in intensity significantly after BAPTA pretreatment in both collagen- and CRP-stimulated platelets. Note that the effects of BAPTA on the 120-kDa region are minor for collagen, although much more apparent for CRP, suggesting that other bands insensitive to BAPTA comigrate with FAK.

Effect of Ionomycin on PKC Activity—Fig. 7a shows that ionomycin, from 500 to 2000 nM, was an effective activator of PKC, determined from the phosphorylation of p47. Higher ionomycin levels caused no further increase in phosphorylation of p47 (data not shown).

Effect of BAPTA-AM on PKC Activity—Fig. 7b shows the effect of BAPTA-AM (20 μM) on platelets activated by collagen (25 μg/ml) or CRP-XL (5 μg/ml). With or without BAPTA loading, both effectors caused marked activation of PKC, indicated by p47 phosphorylation. The action of TPA (not shown) or CRP-XL, was largely insensitive to the presence of BAPTA; only the action of collagen was noticeably attenuated, but PKC...
activity persisted at greater than 50% despite BAPTA loading. This suggests the presence of Ca\(^{2+}\)-sensitive and -insensitive PKC isoforms activated by collagen receptors in human platelets.

**Effect of Ro31-8220 on \([\text{Ca}^{2+}]_i\).**—Fig. 8 shows time courses for the rise in \([\text{Ca}^{2+}]_i\) evoked by collagen (a) or ionomycin (b), with and without pre-incubation of platelets with the PKC inhibitor, Ro31-8220. Inhibition of PKC caused a marked increase in both the peak amplitude and duration of \([\text{Ca}^{2+}]_i\) signals observed under these conditions. Parallel measurement of \([\text{Ca}^{2+}]_i\) using Fura2 showed that calcium signaling was abolished by BAPTA loading (data not shown).

**DISCUSSION**

Our aim in this study was to explore the capacity of the platelet integrin \(\alpha_2\beta_1\) to activate FAK, comparing the efficacy of the synthetic analogue of collagen, CRP-XL, with that of native type I collagen fibers and with the \(\alpha_2\beta_1\)-specific peptide, GFOGER-GPP-XL. Thus, we intended to determine whether \(\alpha_2\beta_1\) acts as a signaling receptor for collagen in platelets, working from the premise that FAK phosphorylation is an event likely to be integrin-dependent in platelets as well as in other cells.

The first part of the present work addresses the role of the collagen receptor \(\alpha_2\beta_1\) in the regulation of FAK. Both collagen and CRP-XL activate FAK, as indicated by its tyrosine phosphorylation state, in a concentration- and time-dependent manner. The failure of antibodies against the \(\alpha_2\) and \(\beta_1\) integrin subunits, which prevent adhesion to collagen (validated as described under "Results") to block FAK activation demonstrates that \(\alpha_2\beta_1\) occupancy by collagen fibers does not regulate FAK. This result contrasts with the proposed general role of \(\beta_1\) integrins in FAK activation (43). These experiments were performed in the presence of micromolar \(\text{Ca}^{2+}\), conditions where the integrin is known to be competent to bind collagen (54). The potency of CRP-XL, which does not bind \(\alpha_2\beta_1\), in stimulating tyrosine phosphorylation of FAK suggests that another collagen receptor, GpVI, initiates FAK activation in platelets.

Recently, we have identified the sequence GFOGER within collagen type I, which binds to the I domain of the integrin \(\alpha_2\) subunit (25). This peptide sequence, in triple-helical conformation, binds platelets in \(\alpha_2\beta_1\)-dependent manner and supports purified \(\alpha_2\beta_1\) binding. By co-crystallization with the recombinant \(\alpha_2\) I domain, we have shown that the E residue of the peptide coordinates the divalent cation in the metal ion-dependent adhesion site of the integrin \(\alpha_2\) subunit (26). This indicates that the peptide properly replicates the \(\alpha_2\beta_1\)-binding properties of collagen. However, even at levels up to 200 \(\mu\)g/ml with or without micromolar \(\text{Ca}^{2+}\) (Fig. 3b), GFOGER-GPP-XL caused no discernible increase in tyrosine phosphorylation of platelet FAK. This shows that neither \(\alpha_2\beta_1\) occupancy nor clustering by the cross-linked peptide is sufficient to activate FAK in platelets in suspension.

The fibrinogen receptor \(\alpha_{IIb}\beta_3\) has attracted most attention as a means of regulating FAK activity in platelets. Collagen
and CRP-XL were added to unstirred suspensions of platelets in the presence of EGTA, conditions where α1bβ3 is not competent and aggregation is not anticipated (57); therefore, we did not expect α1bβ3 to regulate FAK in these experiments. To verify this, we added collagen fibers to platelets stirred in an aggregometer, causing, as anticipated, no significant aggregation. However, despite the presence of EGTA, both CRP-XL and thrombin under similar conditions caused some aggregation, which was highly sensitive to the α1bβ3 antagonist, GR144053F (Fig. 4). This indicates that GR144053F as used here is a good antagonist of α1bβ3 occupancy.

Partial inhibition of FAK phosphorylation by GR144053F in platelets treated with either CRP-XL or thrombin showed that α1bβ3 activation is important in the regulation of FAK, as has been shown previously for thrombin (44). But GR144053F had little effect on the activation of FAK by collagen, consistent with collagen’s failure to cause much aggregation under these conditions. (It should be noted that aggregation is more likely to occur during stirring in the aggregometer than in all other components of the study, where platelets were not stirred for more than a second after the addition of ligand.) The inclusion of both GR144053F and 6F1 (Fig. 5b), to provide simultaneous blockade of α2β1 and α1bβ3, had little effect on FAK tyrosine phosphorylation stimulated by collagen fibers, which is therefore shown to proceed in platelets without the involvement of either integrin under these conditions. Recently, the use of mutant α1bβ3 showed that FAK activation could be dissociated from α1bβ3 occupancy (49), as we propose here for the regulation of FAK by collagen fibers. Integrin-independent activation of FAK has also been reported in platelets activated using immobilized human IgG (48), an event that depends instead on FcγRIIA.

The identity of the collagen receptors responsible for FAK activation remains to be resolved. Our experiments demonstrate that GpVI occupancy alone, resulting from treating platelets with CRP-XL, is not sufficient to elicit full tyrosine phosphorylation of FAK that is independent of α1bβ3. In this respect, CRP-XL shows some similarity to thrombin. Using specific antibodies to cross-link CD36, a candidate receptor along with GpVI, others have discounted CD36 as a regulator of FAK (58), although this technique provides clustering only of CD36 populations rather than of CD36 with other receptors, as we expect will occur with the native collagen fibers used here. Investigation of whether GpVI acts as a co-receptor in regulating FAK in platelets stimulated with collagen fibers, and the possible role of CD36 in these events, must await the development of receptor-specific antagonists.

We sought to identify intracellular signaling events that are involved in the regulation of FAK activity during platelet activation. PKC has been implicated in FAK activation in both platelets (48, 59) and other cells (60, 61) that were adherent to non-collagenous substrates. We found that TPA stimulated tyrosine phosphorylation of FAK in platelet suspensions. However, pretreatment of platelets with the PKC inhibitor, Ro31-8220, virtually abolished tyrosine phosphorylation of FAK caused by collagen or CRP-XL. This suggests that, although direct stimulation of PKC itself is insufficient to cause major stimulation of FAK, PKC is an important mediator of the tyrosine phosphorylation of FAK stimulated by either collagen or CRP-XL.

Next, we showed that [Ca2+]i, is also important in the control of FAK tyrosine phosphorylation, by using ionomycin to elicit Ca2+ mobilization, and BAPTA-AM loading to buffer [Ca2+]i. Ionomycin stimulated tyrosine phosphorylation of FAK, whereas BAPTA-AM completely abolished tyrosine phosphorylation of FAK in platelets stimulated with collagen or CRP-XL, confirming a role for Ca2+. These results contrast with the work of Haimovich et al. (48) who showed that, in IgG-adherent platelets, exposure to BAPTA-AM caused, if anything, increased FAK phosphorylation. The same group reported no effect of BAPTA-AM on FAK phosphorylation in platelets adherent to fibrinogen (59), but in the same paper, they show that BAPTA-AM abolishes the action of thrombin in stimulating FAK in fibrinogen-adherent platelets. A requirement for increased [Ca2+]i, in the regulation of FAK was similarly proposed for epinephrine-stimulated platelet suspensions (59). Possibly, the role of Ca2+ in regulating FAK activity may be ligand-specific.

Ionomycin treatment also activated PKC. To resolve the roles of [Ca2+]i, and PKC, platelets were first preincubated with Ro31-8220 to inactivate PKC and then treated with ionomycin. FAK tyrosine phosphorylation was virtually abolished, as in platelets stimulated with collagen or CRP-XL after PKC blockade. It is important to note that Ro31-8220 enhanced the increase in [Ca2+]i, stimulated by either collagen or ionomycin, very likely as a consequence of inhibiting PKC-dependent Ca2+ ATPases, which export Ca2+ from the cytosol. Reciprocal experiments showed that, although BAPTA blocks FAK phosphorylation, it had little effect on PKC activity. Hence, neither
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elevated [Ca$^{2+}$], or increased PKC activity is sufficient to support FAK phosphorylation, but each is necessary for FAK activation by collagen, CRP-XL, or ionomycin. Such a role for Ca$^{2+}$ has been proposed for endothelial cell FAK activation consequent to spreading on type IV collagen (62). In conclusion, our data suggest that the regulation of platelet FAK by native collagen fibers is independent of integrins, occurring despite blockade of $\alpha_{5}\beta_1$ or $\alpha_{IIb}\beta_3$ or both. The activation of phospholipase C$\gamma$ via GpVI (29, 63) causes Ca$^{2+}$ and PKC signals essential for the regulation of FAK. Yet these signals, together activated by either CRP-XL or thrombin, are not sufficient to elicit full FAK phosphorylation without $\alpha_{IIb}\beta_3$ occupancy. Coordination of signals from $\alpha_{IIb}\beta_3$ and other receptors have been proposed to regulate FAK (34). Our data suggest that collagen, perhaps because it is recognized by different platelet receptor populations in addition to GpVI and $\alpha_{IIb}\beta_3$, is able to bypass the requirement for integrins. The role and identity of these co-receptors for collagen remain to be elucidated.

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