Involvement of proline-rich tyrosine kinase 2 in platelet activation: tyrosine phosphorylation mostly dependent on αIIbβ3 integrin and protein kinase C, translocation to the cytoskeleton and association with Shc through Grb2

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Proline-rich tyrosine kinase 2 (Pyk2) (also known as RAFTK, CAKβ or CADTK) has been identified as a member of the focal adhesion kinase (FAK) family of protein-tyrosine kinases and it has been suggested that the mode of Pyk2 activation is distinct from that of FAK. In the present study we investigated the mode of Pyk2 activation in human platelets. When platelets were stimulated with thrombin, Pyk2, as well as FAK, was markedly tyrosine-phosphorylated, in a manner mostly dependent on αIIbβ3 integrin-mediated aggregation. The residual Pyk2 tyrosine phosphorylation observed in the absence of platelet aggregation was completely abolished by pretreatment with BAPTA/AM [bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl ester]. The Pyk2 phosphorylation was inhibited by protein kinase C (PKC) inhibitors at concentrations that inhibited platelet aggregation. In contrast, direct activation of PKC with the active phorbol ester PMA induced the tyrosine phosphorylation of Pyk2 and FAK but only when platelets were fully aggregated with the exogenous addition of fibrinogen (the ligand for αIIbβ3 integrin). Furthermore, PMA-induced Pyk2 (and FAK) tyrosine phosphorylation was also observed when platelets adhered to immobilized fibrinogen. The activation of the von Willebrand factor (vWF)--glycoprotein Ib pathway with botrocetin together with vWF failed to induce Pyk2 (and FAK) tyrosine phosphorylation. Most Pyk2 and FAK was present in the cytosol and membrane skeleton fractions in unstimulated platelets. When platelets were stimulated with thrombin, both Pyk2 and FAK were translocated to the cytoskeleton in an aggregation-dependent manner. In immunoprecipitation studies, Pyk2, as well as FAK, seemed to associate with Shc through Grb2. With the use of glutathione S-transferase fusion proteins containing Shc-SH2, Grb2-SH2, and Grb2 N-terminal and C-terminal SH3 domains, it was implied that the proline-rich region of Pyk2 (and FAK) binds to the N-terminal SH3 domain of Grb2 and that the phosphotyrosine residue of Shc binds to the SH2 domain of Grb2. Although Pyk2 and FAK have been reported to be differentially regulated in many cell types, our results suggest that, in human platelets, the mode of Pyk2 activation is mostly similar to that of FAK, in terms of αIIbβ3 integrin-dependent and PKC-dependent tyrosine phosphorylation. Furthermore, Pyk2, as well as FAK, might have one or more important roles in post-aggregation tyrosine phosphorylation events, in association with the cytoskeleton and through interaction with adapter proteins including Grb2 and Shc.

Key words: adhesion, focal adhesion kinase, thrombin.

INTRODUCTION

Focal adhesion kinase (FAK) is a non-receptor protein-tyrosine kinase that has a central role in signalling events via integrins [1,2]. FAK co-localizes with integrins at cell--substratum contact sites; its protein-tyrosine kinase activity is enhanced by cellular binding to extracellular matrix proteins [1–4]. Tyrosine phosphorylation of FAK leads to the association of various SH2-containing signalling molecules such as Grb2, phosphoinositide 3-kinase and the Src tyrosine kinase family [5–8]. In addition, the proline-rich domains in the C-terminus can associate with SH3 domains of $p130^{Cas}$ and $p105^{HEF1}$ [9–11]. These complexes and signalling pathways seem to be associated with FAK-mediated cellular functions, including the regulation of cell shape, cell migration, tissue architecture, cell survival, and proliferation [1–4,12].

Proline-rich tyrosine kinase 2 (Pyk2) (also known as RAFTK, CAKβ or CADTK) has been identified as a new member of the FAK family of protein-tyrosine kinases [13–16]; Pyk2 resembles FAK in that it has similar consensus motifs in the central kinase domain, lacks the SH2 and SH3 domains and interacts with Grb2, Src family tyrosine kinases, $p130^{Cas}$ and $p105^{HEF1}$ [11,13,14,17–21]. Because of the high sequence similarity and similar overall organization between FAK and Pyk2, it can be speculated that signalling pathways involving Pyk2 and FAK, especially their responses resulting from integrin activation, are similar. In fact it has been reported that, in B-lymphocytes, megakaryocytes and transfected COS cells, Pyk2 displays integrin-dependent phosphorylation and is localized in focal contacts, as occurs with FAK [19,21]. However, Pyk2 might have a quite unique role (independently of FAK), as follows. Pyk2 tyrosine phosphorylation was not enhanced in rat 3Y1 fibroblasts by fibronectin stimulation [15] and, when overexpressed, full-length Pyk2 was not localized to focal contact sites in chicken fibroblasts [22]. In addition, tyrosine phosphorylation of Pyk2 is regulated in a manner different from that of FAK in PC 12 cells, rat hippocampus, rat aortic smooth-muscle cells and CE cells [13,22–24]. Furthermore, overexpression of

Abbreviations used: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CS, cytoskeleton fraction; CY, cytosol fraction; FAK, focal adhesion kinase; GST, glutathione S-transferase; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MS, membrane skeleton fraction; PKC, protein kinase C; Pyk2, proline-rich tyrosine kinase 2; TRAP, thrombin receptor-activating peptide; vWF, von Willebrand factor.

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Pyk2 in both fibroblast and epithelial cell lines reportedly leads to cellular apoptosis, whereas FAK is required for cell survival [25]. Finally, Pyk2, but not FAK, was shown to be involved in Ca^{2+}-dependent signalling events, leading to ion channel regulation and mitogen-activated protein kinase (MAPK) activation [13].

It has been reported that, in platelets, tyrosine phosphorylation of FAK is a relatively late event in platelet activation in comparison with that of Src and Syk [26]. FAK tyrosine phosphorylation has been shown to be dependent on protein kinase C (PKC), actin polymerization and irreversible aggregation [27,28]. Furthermore, FAK translocates to the cytoskeleton in an aggregation-dependent manner [29] and undergoes proteolytic cleavage, leading to termination of the kinase activation [29]. These findings indicate that FAK regulates post-aggregation tyrosine phosphorylation events through αIIbβ3 integrin (glycoprotein IIb/IIIa) in platelets; αIIbβ3 is the most abundant platelet adhesion receptor, which binds to fibrinogen, fibronectin, vitronectin and von Willebrand factor (vWF) and mediates platelet aggregation, firm adhesion and spreading [30,31].

Although many observations have been reported of the regulation of FAK tyrosine phosphorylation in platelets, little is known about the mode of Pyk2 activation in platelets. Raja et al. [32] have, for the first time, reported an analysis of Pyk2 tyrosine phosphorylation in platelet activation. Pyk2 was shown to be tyrosine-phosphorylated during the early phase of platelet activation by a mechanism independent of αIIbβ3 integrin and platelet aggregation [32]. Furthermore, Pyk2 tyrosine phosphorylation was abolished by the preincubation of platelets with PKC inhibitors [32]. Accordingly, it was concluded that Pyk2 tyrosine phosphorylation is mediated by PKC but not by αIIbβ3-mediated platelet aggregation [32]. In the present study we examined and compared in detail the involvement of Pyk2 and FAK during platelet activation. We found that Pyk2 phosphorylation (as well as FAK) was mostly dependent on αIIbβ3 outside-in signalling and on PKC activation. In addition, both Pyk2 and FAK translocate into cytoskeleton in an aggregation-dependent manner and associate with adapter proteins, including Grb2 and Shc. These results suggest that Pyk2, as well as FAK, regulates post-aggregation tyrosine phosphorylation events, in association with the cytoskeleton and through interaction with adapter proteins, including Grb2 and Shc.

MATERIALS AND METHODS

Materials

Botrocetin and vWF [33], and PMA4 $F(ab)_2$ [34] were kindly provided by Dr Y. Fujimura (Nara Medical University, Nara, Japan) and Dr T. Hato (Ehime University School of Medicine, Ehime, Japan) respectively. Ro31-8220, a specific PKC inhibitor [35], was a gift from Roche Products (Welwyn Garden City, Herts., U.K.). The following materials were obtained from the indicated suppliers: thrombin (Green Cross, Osaka, Japan); collagen (Hormon-Chemie, Munich, Germany); SFLLRN [thrombin receptor-activating peptide (TRAP)] (Sawaday Technology, Tokyo, Japan); GRGDS peptide (Peptide Institute, Osaka, Japan); anti-Pyk2 polyclonal antibody, anti-FAK monoclonal antibody (mAb) (2A7, used for immunoprecpitation) and anti-phosphotyrosine mAb (4G10) (Upstate Biotechnology, Lake Placid, NY, U.S.A.); anti-Pyk2 mAb, anti-FAK mAb (clone 77, used for immunoblotting), anti-Shc mAb, anti-Shc polyclonal antibody, anti-Grb2 mAb, and anti-phosphotyrosine mAb (PY20) (Transduction Laboratories, Lexington, KY, U.S.A.); control mouse IgG (Zymed Laboratories, San Francisco, CA, U.S.A.); PMA and fibrinogen (Sigma Chemical Co., St Louis, MO, U.S.A.); glutathione–Sepharose 4B and Protein A–Sepharose CL-4B (Pharmacia Biotech, Uppsala, Sweden).

Platelet preparation and aggregation monitoring

Platelet-rich plasma was prepared as previously described [36]. The platelets were washed and resuspended in a buffer containing 138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 1 mg/ml glucose and 20 mM Hepes, pH 7.4. Just before centrifugation of platelet suspensions, a 15% volume of acid-citrate/dextrose A solution and 0.1 μM prostaglandin I_2 was added to inhibit platelet activation. The final platelet suspensions were adjusted to 10^9 cells/ml and supplemented with 1 mM $CaCl_2$ unless stated otherwise. When intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentrations were measured, 100 μM EGTA was used instead of $CaCl_2$. Platelet aggregation was monitored (at 37 °C under continuous stirring at 1000 rev./min) by measuring light transmission with the use of an AA-100 platelet aggregation analyser (Sysmex, Kobe, Japan). The instrument was calibrated with a platelet suspension for zero light transmission and with a buffer for 100% transmission.

Under the above conditions, αIIbβ3-mediated platelet aggregation was elicited by stimulants capable of inducing a release reaction; fibrinogen, released from platelet α granules, binds to activated αIIbβ3 in the presence of extracellular Ca^{2+}, leading to platelet aggregation [37]. In fact, when platelets were stimulated with thrombin (see Figure 1A, left panel), collagen or TRAP (results not shown), marked aggregation of platelets was observed without the addition of fibrinogen. To obtain platelets stimulated with these agonists but not aggregated, the cells were preincubated with 200 μM GRGDS (a competitive antagonist to inhibit the interaction between fibrinogen and αIIbβ3) [31] and 1 mM EDTA (instead of Ca^{2+}) for 5 min; platelet aggregates were not formed under these conditions (see Figure 1A, right panel). In contrast, when platelets were stimulated with PMA, only very weak platelet aggregation was observed, in spite of marked PKC activation, which was monitored by pleckstrin phosphorylation (see the Platelet PKC activation section). This might be because PMA is not a potent stimulant for release reactions and insufficient fibrinogen (for platelet aggregation) was released from the platelet granules [38]. Accordingly, 400 μg/ml of fibrinogen was added 5 min before stimulation to observe PMA-induced platelet aggregation, whereas 200 μM GRGDS was used to block platelet aggregation completely (see Figure 3A).

Immunoprecipitation

Washed platelets were stimulated as indicated, and reactions were terminated with an equal volume of ice-cold 2 × lysis buffer [2% (v/v) Triton X-100/100 mM Tris/HCl (pH 7.2)/2 mM EGTA/2 mM Na_3VO_4/1 mM PMSF/100 μg/ml leupeptin]. All subsequent immunoprecipitation steps were performed at 4 °C. After sonication, the lysates were centrifuged at 15000 ***g*** for 5 min and then precleared with Protein A–Sepharose CL-4B. The resultant supernatants were incubated for 2 h with the antibody indicated. Protein A–Sepharose CL-4B was then added and incubated for a further 1 h. The Sepharose beads were washed three times with 1 × lysis buffer. The samples were then solubilized with an SDS sample buffer and used for immunoblotting, as described below.

Immunoblotting

The proteins were resolved by SDS/PAGE and then electrophoretically transferred to a PVDF membrane. The membranes were blocked with 1% (w/v) BSA in PBS. After extensive washing with PBS containing 0.4% (v/v) Tween 80, the immunoblots were incubated for 2 h with anti-Pyk2 mAb (0.25 μg/ml), anti-FAK mAb (0.25 μg/ml), anti-phosphotyrosine mAb (1 μg/ml of PY20 plus 1 μg/ml of 4G10), anti-Shc mAb (0.25 μg/ml) or anti-Grb2 mAb (0.05 μg/ml). Antibody binding was detected with peroxidase-conjugated goat anti-mouse IgG and detected with enhanced chemiluminescence reaction reagents (ECL®; Amersham, Little Chalfont, Bucks., U.K.). Where indicated, levels of tyrosine phosphorylation were quantified with a PDI400oe Scanner and with Quantity One 2.5a software for Macintosh. For reprobing with other antibodies, the antibody binding to the PVDF membrane was removed with a stripping buffer [2% (w/v) SDS/62.5 mM Tris/HCl (pH 6.8)/100 μM 2-mercaptoethanol] at 60 °C for 30 min. After washing twice with PBS containing 0.4% (v/v) Tween 80, the membranes were blocked with 1% (w/v) BSA and reprobed with the antibody indicated.

Platelet adhesion assay

Platelet adhesion to fibrinogen was assayed in 100 mm dishes (Becton Dickinson Labware, Franklin Lakes, NJ, U.S.A.). The dishes were coated with 200 μg/ml fibrinogen for 16 h at room temperature, washed twice with PBS, then blocked with 1 mg/ml BSA for 1 h. Washed platelets (3 ml; 2×10^8/ml) were placed on the fibrinogen-coated dishes for 1 h at 37 °C. After being washed twice with PBS, adherent platelets wer lysed with 1 ml of $1 \times$ lysis buffer (see the Immunoprecipitation section) with the aid of a cell scraper. As a control, non-adherent platelets from BSA-coated dishes were used. The protein concentration of each lysate was measured; 0.5 mg of protein was used for immunoprecipitation.

Fractionation of platelets

Subcellular fractionation of platelets was performed with a modification of the method described previously [39]. Reactions were terminated with an equal volume of $2 \times$ lysis buffer (see the Immunoprecipitation section above). All subsequent steps were performed at 4 °C. The cytoskeleton fraction (CS) was isolated by centrifugation of the lysate at 15000 ***g*** for 5 min. The membrane skeleton fraction (MS) was isolated from the resultant supernatant by centrifugation at 100000 ***g*** for 3 h with a Himac CS 100FX (Hitachi Koki Co., Tokyo, Japan). For the analysis of the cytosol fraction (CY), Pyk2 or FAK was immunoprecipitated with a specific antibody from the Triton X-100-soluble fractions from the 100000 ***g*** supernatant. The proteins obtained from CS, MS and CY were washed three times with $1 \times$ lysis buffer and solubilized with 75 μl of $1 \times$ SDS sample buffer and boiled for 3 min. The proteins of these fractions were detected by immunoblotting, as described above.

Glutathione S-transferase (GST) fusion protein production and binding studies

Plasmids encoding GST fusion proteins containing the SH domains of Grb2 [40] and the SH2 domain of Shc [41] were kindly provided by Dr T. Takenawa (University of Tokyo, Tokyo, Japan) and Dr T. Pawson (Mount Sinai Hospital, Toronto, ON, Canada) respectively. All fusion protein constructs were transformed into *Escherichia coli* for protein production. The GST fusion proteins were purified on a glutathione–Sepharose column by affinity chromatography in accordance with the manufacturer's recommendations (Pharmacia Biotech). For the binding experiments, the platelet lysates were precleared with glutathione--Sepharose 4B, then mixed with 20 μg of GST fusion protein and incubated for 1 h at 4 °C on a rotatory shaker. Glutathione--Sepharose 4B beads (40 μl) were added to preabsorb the protein complex. After incubation for 1 h, the beads were centrifuged and washed three times with $1 \times$ lysis buffer. The bound proteins were eluted with $1 \times$ SDS sample buffer and boiled for 3 min. The proteins were separated by SDS/PAGE and immunoblotted as described above.

Measurement of $[Ca^{2+}]_i$

Measurement of $[Ca^{2+}]_i$ was performed with the use of the Ca^{2+}-sensitive fluorophore fura-2 as described previously [42]. The platelet concentration was adjusted to 2×10^8 cells/ml and 100 μM EGTA was added. The $[Ca^{2+}]_i$ values were determined from the ratio of the fura-2 fluorescence intensity at 340 nm with excitation at 380 nm.

Platelet PKC activation

PKC activation in intact platelets was evaluated by pleckstrin (p47) phosphorylation, as described previously [43].

RESULTS

Dependence of Pyk2 and FAK tyrosine phosphorylation on αIIbβ3-mediated platelet aggregation

It has been established that the tyrosine phosphorylation of FAK is dependent on αIIbβ3-mediated platelet aggregation [26–28]. In the present study we first examined the involvement of this integrin in platelet Pyk2 tyrosine phosphorylation. When platelets were stimulated and aggregated with thrombin (Figure 1A), Pyk2, as well as FAK, was gradually tyrosine-phosphorylated (Figure 1B); the phosphorylation of Pyk2 slightly preceded that of FAK. When platelet aggregation was suppressed by pretreatment with EDTA plus GRGDS (Figure 1A) (see the Materials and methods section), the tyrosine phosphorylation of both Pyk2 and FAK was suppressed (Figures 1B and 1C). Aggregation-dependent phosphorylation of Pyk2 and FAK was also observed when platelets were challenged with collagen or TRAP (results not shown). Although the stimulated phosphorylation of FAK is entirely dependent on αIIbβ3-mediated platelet aggregation, a residual degree of Pyk2 phosphorylation was detected in the absence of aggregation (Figures 1B and 1C).

We next characterized the factor(s) involved in the residual Pyk2 phosphorylation. In condition under which platelet aggregation was completely abolished (see above), pretreatment with BAPTA/AM [bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid acetoxymethyl ester], an intracellular Ca^{2+} chelator, was found to inhibit the remaining Pyk2 tyrosine phosphorylation (induced by thrombin) (Figure 2A). In addition, the Ca^{2+} ionophore A23187 (0.1 μM) induced a slight tyrosine phosphorylation of Pyk2 (Figure 2B). These results suggest that weak Pyk2 tyrosine phosphorylation can be induced independently of platelet aggregation through enhanced levels of cytosolic Ca^{2+}.

Blockage by PKC inhibitors of both platelet aggregation and Pyk2 tyrosine phosphorylation

We [44] and others [32] have reported that Pyk2 tyrosine phosphorylation is mediated by PKC activation in platelets,

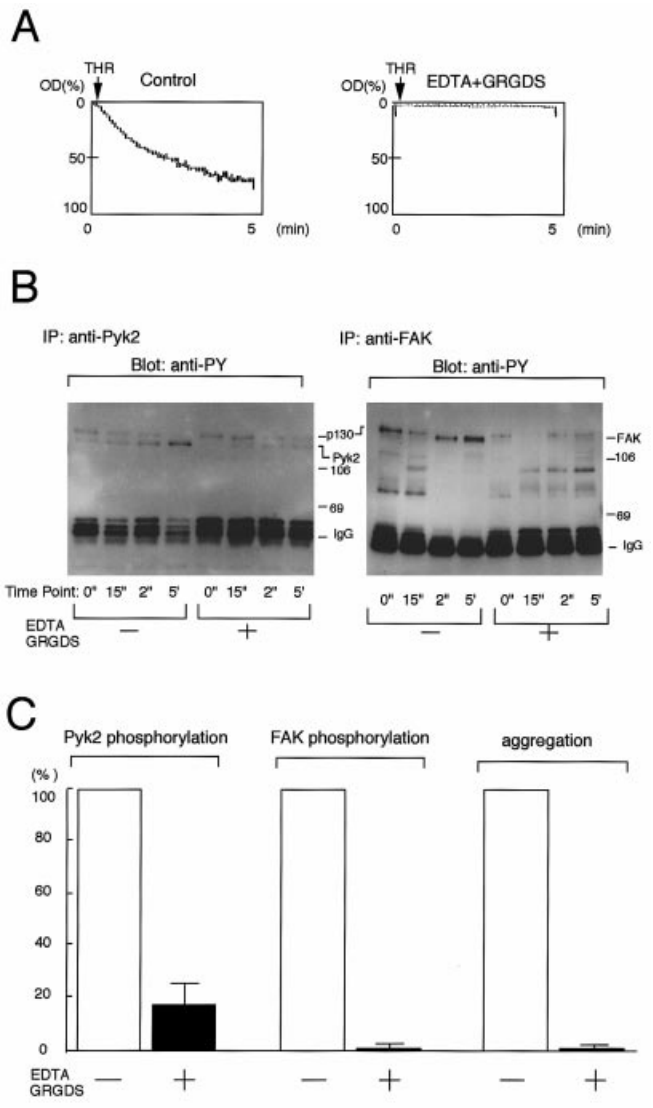

Figure 1 Effects of EDTA plus GRGDS on thrombin-induced platelet aggregation and tyrosine phosphorylation of Pyk2 and FAK

Platelets pretreated without or with 1 mM EDTA plus 200 μM GRGDS peptide for 5 min were stimulated with 1 unit/ml thrombin (THR) for the indicated durations. (**A**) Platelet aggregation was monitored turbidometrically. (**B**) Platelet lysates were immunoprecipitated (IP) with anti-Pyk2 polyclonal antibody or anti-FAK mAb, resolved by SDS/PAGE [8% (w/v) gel], then immunoblotted with anti-phosphotyrosine (anti-PY) mAb. The locations of Pyk2 and FAK are indicated at the right. We often noticed 130 kDa tyrosine-phosphorylated protein (p130) in immunoprecipitates with anti-Pyk2 or FAK (see also Figure 4). Because the protein band also appeared in immunoblots with control mouse IgG (results not shown), this was considered to be non-specific. (**C**) Levels of Pyk2 (left panel) and FAK (middle panel) tyrosine phosphorylation were quantified in platelets challenged with 1 unit/ml thrombin for 2 min, as described in the Materials and methods section. Under the conditions used, it was confirmed that the combined addition of EDTA and GRGDS peptides abolished thrombin-induced platelet aggregation (right panel). Results are expressed as percentages of control values obtained from thrombin-stimulated platelets (without pretreatment). Columns and error bars represent means $\pm$ S.D. ($n = 3$). The locations of molecular mass markers (in kDa) are indicated by '69' and '106'.

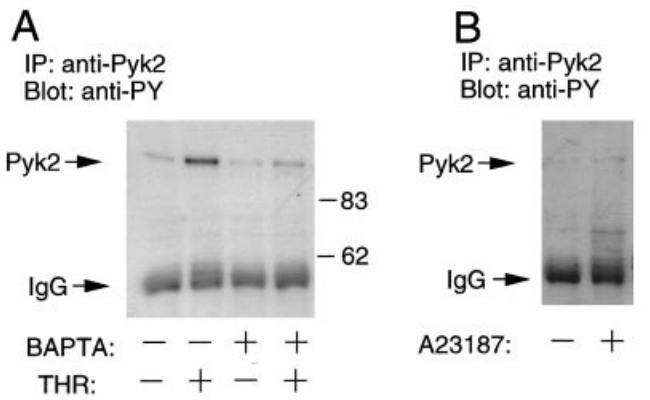

Figure 2 Involvement of Ca^{2+} mobilization in aggregation (αIIbβ3 integrin)-independent Pyk2 tyrosine phosphorylation

In the following experiments, platelets were pretreated with 200 μM GRGDS peptide and 1 mM EDTA to inhibit platelet aggregation (see Figure 1A). (**A**) Platelets were incubated without or with 50 μM BAPTA/AM for 15 min, then challenged or not with 1 unit/ml thrombin (THR) for 2 min. The locations of molecular mass markers (in kDa) are indicated by '62' and '83'. (**B**) Platelets were stimulated with 100 nM A23187 for 2 min or not. The immunoprecipitates (IP) with anti-Pyk2 polyclonal antibody or anti-FAK mAb were resolved by SDS/PAGE [8% (w/v) gel], then immunoblotted with anti-phosphotyrosine (anti-PY) mAb. The results are representative of three experiments.

because pretreatment with PKC inhibitors results in its inhibition. As expected from the previous studies [28,32,34], thrombin-induced PKC activation (as evaluated by pleckstrin phosphorylation) (results not shown) and the tyrosine phosphorylation of FAK (results not shown) and Pyk2 (Figure 3A) were markedly inhibited by preincubation of platelets with staurosporine or Ro31-8220; the former is a potent (but non-specific) protein kinase inhibitor [45], whereas the latter is a specific PKC inhibitor [35]. However, thrombin-induced platelet aggregation was also inhibited under these conditions (Figure 3B). This is consistent with the observations that PKC activation is a key mediator of integrin inside-out signalling [46] and that it is involved in the process of agonist-induced platelet aggregation [47]. Accordingly, the involvement of PKC in Pyk2 (and FAK) tyrosine phosphorylation, on the basis of the results of the PKC inhibitors, might have been overestimated owing to their inhibitory effects on platelet aggregation.

PMA-induced tyrosine phosphorylation of Pyk2 and FAK

To assess further the involvement of PKC in the tyrosine phosphorylation of Pyk2 and FAK, we examined the effects of the active phorbol ester PMA, which directly activates PKC by acting as a substitute for diacylglycerol [48]. When platelets were treated with PMA, marked PKC activation was observed in both the absence and the presence of the GRGDS peptide; PMA induced a weak aggregation in the absence of this peptide, but not at all in its presence (results not shown). As expected from previous studies [27,28], PMA failed to induce FAK tyrosine phosphorylation (Figure 4B) when the platelet aggregation reaction was blocked by GRGDS (Figure 4A, left panel) but did induce its phosphorylation (Figure 4B) when platelets were aggregated by the addition of fibrinogen (Figure 4A, right panel) (see also the Materials and methods section). Similar findings were observed when Pyk2 tyrosine phosphorylation was examined; PMA induced Pyk2 tyrosine phosphorylation only when the aggregation reaction was induced by the addition of fibrinogen (Figure 4B). Consequently, PKC activation alone (without αIIbβ3 outside-in signalling) is not enough to induce the tyrosine phosphorylation of Pyk2 (and FAK).

We next attempted to determine whether fibrinogen–αIIbβ3 interaction is sufficient to phosphorylate Pyk2 (in the absence of PKC activation). We used PMA4 $F(ab)_2$, which induces a direct

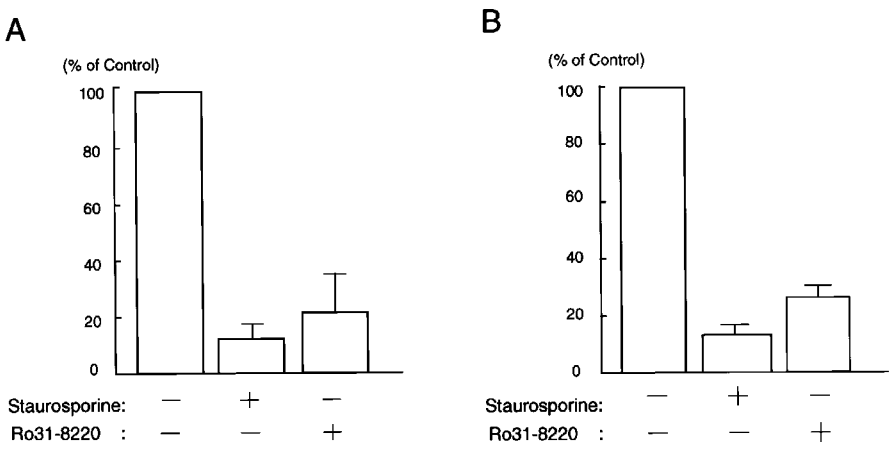

Figure 3 Effects of PKC inhibitors on thrombin-induced platelet Pyk2 tyrosine phosphorylation and aggregation

Platelets were pretreated without or with 1 μM staurosporine or 10 μM Ro31-8220 for 5 min, then stimulated with 1 unit/ml thrombin for 2 min. (**A**) Levels of Pyk2 tyrosine phosphorylation were quantified by densitometry. (**B**) Levels of platelet aggregation were measured by light transmission. Results are expressed as percentages of control values obtained from thrombin-stimulated cells (without pretreatment). Columns and error bars represent means $\pm$ S.D. ($n = 3$).

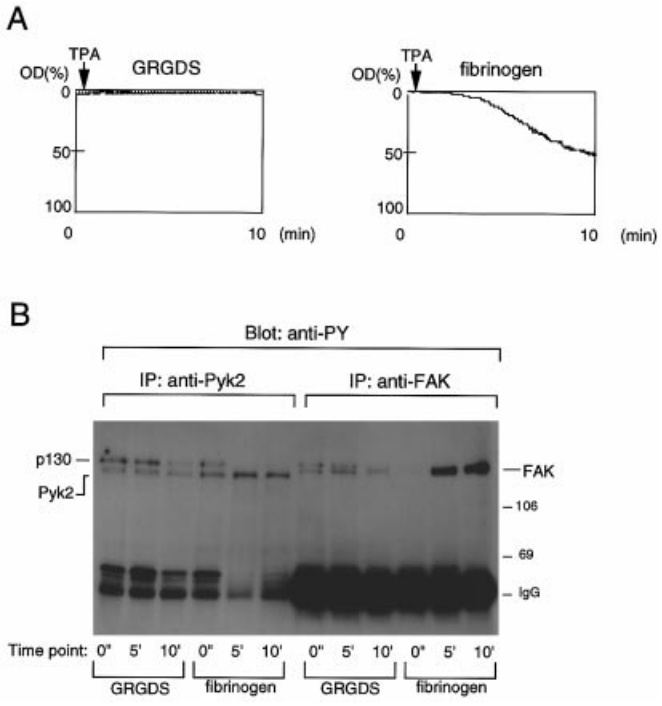

Figure 4 PMA-induced tyrosine phosphorylation of Pyk2 and FAK in platelets

Platelets were pretreated with 200 μM GRGDS peptide or 400 μg/ml fibrinogen for 5 min, then stimulated with 1 μM PMA (TPA) for the indicated durations. (**A**) Platelet aggregation was monitored by light transmission. (**B**) The immunoprecipitates (IP) with anti-Pyk2 polyclonal antibody or anti-FAK mAb were resolved by SDS/PAGE [8% (w/v) gel], then immunoblotted with anti-phosphotyrosine (PY) mAb. The results are representative of three experiments. The locations of molecular mass markers (in kDa) are indicated by '69' and '106'.

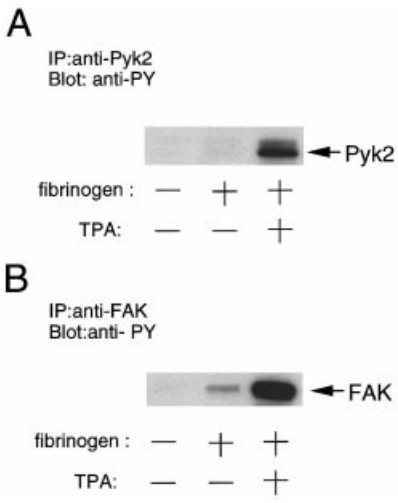

Figure 5 Tyrosine phosphorylation of Pyk2 and FAK induced by adhesion of platelets to immobilized fibrinogen

Platelets treated without or with 10 nM PMA (TPA) were placed on immobilized fibrinogen for 1 h as described in the Materials and methods section. Platelets recovered from BSA-coated dishes were used as a control. The cell lysates were immunoprecipitated (IP) with anti-Pyk2 polyclonal antibody (**A**) or anti-FAK mAb (**B**), then immunoblotted with anti-phosphotyrosine (anti-PY) mAb. The results are representative of three experiments.

change in the extracellular domains of αIIbβ3 integrin and exposes the fibrinogen-binding site without PKC activation [34]. As reported [34], PMA4 $F(ab)_2$ induced platelet aggregation in the presence of fibrinogen (results not shown). Under the conditions used, neither Pyk2 nor FAK was tyrosine-phosphorylated (results not shown). Accordingly, both αIIbβ3-mediated signalling and PKC activation might be necessary for the tyrosine phosphorylation of Pyk2 (and FAK) in platelets.

We confirmed the requirement of both αIIbβ3-mediated outside-in signalling and PKC activation for Pyk2 (and FAK) tyrosine phosphorylation with the use of a platelet adhesion assay. Platelet adhesion to immobilized fibrinogen by itself did not lead to Pyk2 tyrosine phosphorylation, whereas slight FAK tyrosine phosphorylation was observed under the same conditions (Figure 5). Tyrosine phosphorylation of Pyk2 and FAK induced by adhesion was markedly augmented when platelets were treated with PMA (Figure 5).

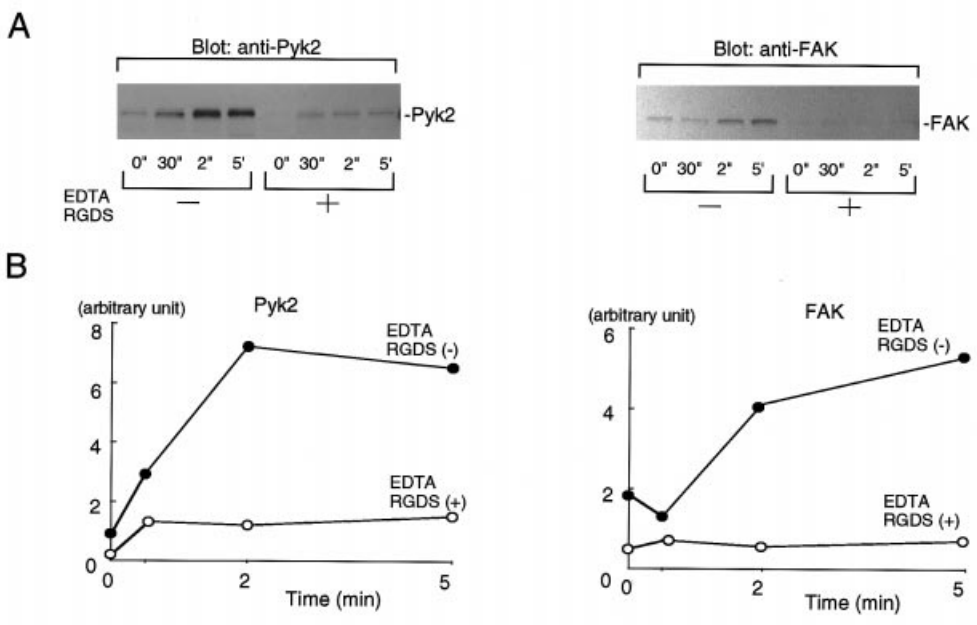

Figure 6 Aggregation-dependent translocation of Pyk2 and FAK into the cytoskeleton in thrombin-activated platelets

Platelets pretreated for 5 mins without or with 1 mM EDTA plus 200 μM GRGDS peptide were stimulated with 1 unit/ml thrombin for the indicated durations. Translocation of Pyk2 or FAK into the cytoskeleton was measured as described in the Materials and methods section. (**A**) Cytoskeletal samples were subjected to SDS/PAGE and probed with anti-Pyk2 mAb or anti-FAK mAb. (**B**) The levels of Pyk2 and FAK were quantified by densitometry. The results are representative of three experiments.

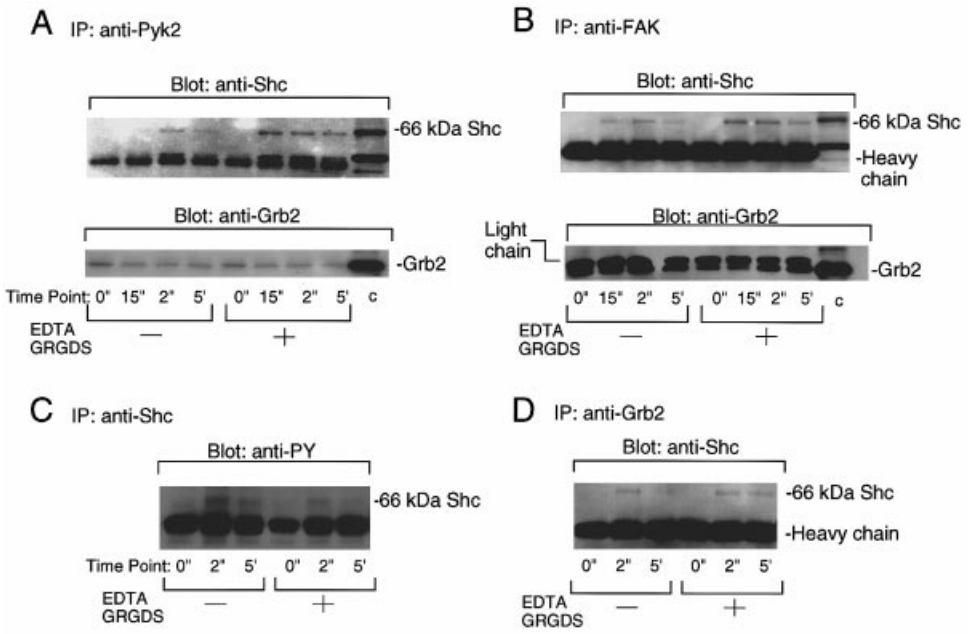

Figure 7 Association of Shc with Pyk2 and FAK through Grb2 in thrombin-activated platelets

Platelets pretreated without or with 1 mM EDTA plus 200 μM GRGDS peptide for 5 min were stimulated with 1 unit/ml thrombin for various durations. Immunoprecipitates (IP) obtained with anti-Pyk2 polyclonal antibody (**A**), anti-FAK mAb (**B**), anti-Shc polyclonal antibody (**C**), or anti-Grb2 mAb (**D**), along with whole cell lysate (c), were subjected to SDS/PAGE and immunoblotted with the antibody indicated. The results are representative of three experiments.

Failure of GPIb–vWF interactions to induce the tyrosine phosphorylation of Pyk2 and FAK

Thrombus formation is initiated by the adhesion of platelets to the sites of vascular injuries, which involves the exposure of subendothelial extracellular matrices and an interaction between vWF and its platelet membrane receptor, GPIb [49]. We therefore examined the tyrosine phosphorylation of Pyk2 and FAK in platelets activated through vWF–GPIb interactions. For this purpose, platelets were stimulated with botrocetin, a snake venom

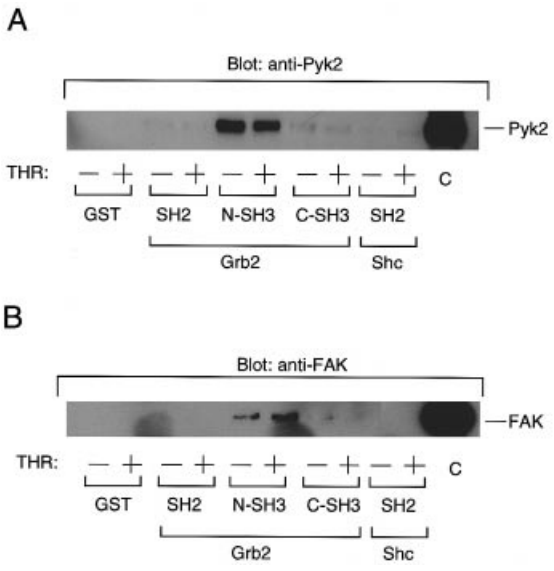

Figure 8 Association of Pyk2 or FAK with GST fusion proteins containing the SH domains of Grb2 and Shc

Platelets were stimulated without (−) or with (+) 1 unit/ml thrombin (THR) for 2 min. The cell lysates were incubated with GST fusion protein containing the SH2 domain, the N-terminal SH3 domain or the C-terminal SH3 domain of Grb2, or the SH2 domain of Shc, then incubated with glutathione--Sepharose 4B. Proteins associated with GST fusion protein were subjected to SDS/PAGE and probed with anti-Pyk2 mAb (**A**) or anti-FAK mAb (**B**). The results are representative of three experiments.

purified from *Bothrops jararaca* [33], in the presence of vWF; this venom induces vWF–GPIb interactions and platelet activation by facilitating the binding of vWF to GPIb [33]. When platelets pretreated with the GRGDS peptide (to inhibit vWF–αIIbβ3 interaction [30,31]) were challenged with vWF and then botrocetin, tyrosine phosphorylation of Pyk2 or FAK was not observed (results not shown). Under these conditions, platelet aggregation (agglutination) induced by vWF–GPIb interactions was observed (results not shown). Accordingly, the specific signal originating from αIIbβ3, but not GPIb, seems to be necessary for the tyrosine phosphorylation of Pyk2 and FAK.

Subcellular distribution of Pyk2 and FAK in platelets

We next examined the subcellular distribution of Pyk2 and FAK, with special emphasis on cytoskeletal reorganization. Most Pyk2 and FAK was present in CY and MS in unstimulated platelets (results not shown). When platelets were stimulated with thrombin (and aggregated), not only FAK but also Pyk translocated to the cytoskeleton in a time-dependent manner (Figure 6). The translocation of Pyk2 (and FAK) to the cytoskeleton was dependent mostly on αIIbβ3-mediated platelet aggregation (Figure 6). A residual degree of Pyk2 (but not FAK) translocation was observed in the absence of aggregation; the reason for this remains to be solved. A marked decrease in the amount of FAK in CY was also observed; this preceded FAK translocation to the cytoskeleton (results not shown); it might be related to the previously reported fact that FAK undergoes proteolysis in thrombin-activated platelets [29]. In contrast, the amount of Pyk2 in CY did not decrease under identical conditions (results not shown).

Association of Shc with Pyk2 and FAK through Grb2 in activated platelets

Several proteins, including the Src-family tyrosine kinases Grb2, p130^{Cas} and p105^{HEF1}, have been reported to be associated with Pyk2 [11,18–20] and FAK [7–10] in several cells. We examined signalling or docking proteins that associate with Pyk2 or FAK in activated platelets. Cell lysates from platelets stimulated with thrombin were immunoprecipitated and then immunoblotted with a variety of antibodies. We observed a specific association of both Pyk2 and FAK with Grb2 and Shc. Grb2 was constitutively associated with Pyk2 and FAK (Figures 7A and 7B). In contrast, the 66 kDa Shc interacted with Pyk2 and FAK after activation with thrombin (Figures 7A and 7B). Furthermore, Shc was found to be tyrosine-phosphorylated and to associate with Grb2 in a stimulation-dependent manner (Figures 7C and 7D). All these associations were observed in both the presence and the absence of platelet aggregation (Figure 7). Because both Pyk2 and FAK tyrosine phosphorylation were suppressed by the inhibition of platelet aggregation (Figure 1), the possibility that phosphotyrosine residues in Pyk2 or FAK bind to the SH2 domain of Shc is remote. The possibility of an interaction between the phosphotyrosine residue in Shc and Pyk2 or FAK is also remote because of a lack of a SH2 domain or other phosphotyrosine-binding motifs in Pyk2 or FAK [1,2,13,14]. Accordingly, it is most probable that Shc becomes associated with Pyk2 or FAK after stimulation through Grb2; tyrosine-phosphorylated Shc might interact with Grb2, which constitutively associates with Pyk2 or FAK. To confirm this hypothesis, GST fusion proteins containing Shc-SH2, Grb2-SH2 and Grb2 N-terminal and C-terminal SH3 domains were used for precipitation of the associated proteins. As expected, the N-SH3 domain of Grb2 was found to specifically and constitutively with Pyk2 (Figure 8A) and FAK (Figure 8B). These results indicate that the proline-rich region of Pyk2 or FAK binds to the N-terminal SH3 domain of Grb2 and that the phosphotyrosine residue of Shc binds to the SH2 domain of Grb2.

DISCUSSION

The aim of this study was to examine the mode of Pyk2 phosphorylation (activation) in platelets. It is well established that FAK is the essential component in focal adhesions in various cells, including platelets [1–4,10,12]. Although Pyk2 is a non-receptor tyrosine kinase belonging to the FAK gene family, its activation mechanism and involvement in cellular functions have been reported to be distinct from those of FAK [13,15,22–24]. In addition, in platelets, tyrosine phosphorylation of Pyk2 [32], but not FAK [27,28], was reportedly independent of αIIbβ3 integrin. However, in the present study we found that tyrosine phosphorylation of Pyk2 was mostly dependent on αIIbβ3-mediated outside-in signalling. When platelet aggregation was inhibited, only a slight Pyk2 phosphorylation was observed, which was probably induced by an elevation of $[Ca^{2+}_i]$. Raja et al. [32] have reported that thrombin-induced Pyk2 tyrosine phosphorylation was not affected in the absence of stirring or by the pretreatment of platelets with an RGDS peptide or anti-αIIbβ3 mAb. However, platelet aggregation was not monitored in their study. Under their conditions, we found that thrombin-induced platelet aggregation was inhibited only partly (results not shown). Platelet aggregation was inhibited completely only when extracellular Ca^{2+} depletion and GRGDS treatment were performed simultaneously. We confirmed that thrombin-induced aggregation was completely suppressed (when it should be) under our conditions, by simultaneously monitoring light trans-

mission. This might explain the discrepancy between our results and those of Raja et al.

Pyk2 is tyrosine-phosphorylated and activated in response to various stimuli that activate PKC; the dependence of Pyk2 phosphorylation on PKC in several systems [13,23,50] has been postulated. In platelets, PKC involvement in tyrosine phosphorylation of Pyk2, as well as FAK, was reported with the use of PKC inhibitors [28,32,44]. However, the effects of these inhibitors might have been overestimated as a results of their inhibitory effects on αIIbβ3-mediated platelet aggregation (see Figure 3). In the present study we found that PKC activation by PMA alone failed to induce the tyrosine phosphorylation of Pyk2 (or FAK) in platelets; the simultaneous induction of platelet aggregation with the addition of fibrinogen resulted in the phosphorylation of Pyk2 (and FAK). In contrast, only fibrinogen binding to αIIbβ3 integrin (in the absence of PKC activation) with the use of PMA4 $F(ab)_2$ did not lead to the tyrosine phosphorylation of Pyk2 (or FAK). Furthermore, αIIbβ3 binding to immobilized fibrinogen (platelet adhesion) resulted in Pyk2 tyrosine phosphorylation only when platelets were stimulated with PMA. These results indicate that both PKC activation and αIIbβ3-mediated outside-in signalling are required for Pyk2 (and FAK) tyrosine phosphorylation in platelets.

GPIb is a unique adhesive polypeptide unrelated in structure to the members of the integrin, selectin or Ig superfamilies; it is a type I membrane-spanning polypeptide that belongs to the leucine-rich motif family [49]. GPIb has a crucial role in the first stage of the interaction of platelets with exposed subendothelium via interaction with vWF [49]. We have shown previously that GPIb–vWF interaction mediates the tyrosine phosphorylation of Syk and Shc and the translocation of Src and Lyn to cytoskeletal fractions. In addition, the tyrosine kinase associated with GPIb serves at the most proximal step in the signal transduction pathway leading to other tyrosine kinase-related intracellular signals [33]. In the present study, however, the GPIb–vWF interaction failed to induce the phosphorylation of Pyk2 (and FAK). These results might support our idea that specific signalling from αIIbβ3 (but not GPIb) is necessary for induction of Pyk2 (and FAK) tyrosine phosphorylation, in addition to PKC activation.

After platelet activation, many proteins, including tyrosine kinases, shift to cytoskeleton fractions [29,51]. Although the precise roles of the cytoskeletal association of tyrosine kinases remain largely unknown [51], they might contribute to stabilizing platelet aggregates or to inducing clot retraction [51]. In the present study, most of Pyk2, as well as FAK, was found to be present in cytosol and the membrane skeleton in resting states, and to translocate to the cytoskeleton in an aggregation-dependent manner. Not only FAK but also Pyk2 might be important in post-aggregation events in association with the cytoskeleton.

A similarity between Pyk2 and FAK functional responses was also shown in their interaction with adapter proteins: we showed that Grb2 constitutively associates with both Pyk2 and FAK through the N-terinal SH3 domain and interacts with tyrosine-phosphorylated Shc after platelet activation. Grb2 and Shc are adapter molecules involved mainly in the Ras–MAPK cascade [52]; these molecules reportedly interact with FAK and Pyk2 [1,2,5,19,20]. However, the precise role of Grb2 and Shc in Pyk2- and FAK-mediated cell function remains to be established [1,2,5,19,20]. Although the physiological implications for the interaction of Pyk2 and FAK with Grb2 and Shc in platelets also remain to be clarified, it should be noted that the modes of interaction with adapter molecules are similar for Pyk2 and FAK.

In summary, our results suggest that both Pyk2 and FAK are activated in a manner dependent on PKC and αIIbβ3 outside-in signalling, translocate into the cytoskeleton in an aggregation-dependent manner, and associate with Shc through Grb2. Because of the high degree of sequence similarity between Pyk2 and FAK [13–15], it is surprising that these kinases are differentially regulated in many other cell types: (1) Pyk2 was activated by a variety of soluble agonists that elevated $[Ca^{2+}]_i$ [13,15,16,23], whereas FAK is responsive mainly to signals from integrin adhesion [1–4]; 2) FAK is required for cell survival, whereas Pyk2 induces apoptosis [25]. Our findings that both Pyk2 and FAK are functionally overlapping in platelet activation might indicate the unique roles of the FAK family tyrosine kinases in these anucleate cells.

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