Platelet α Ib- β 3 integrin engagement induces the tyrosine phosphorylation of Cbl and its association with phosphoinositide 3-kinase and Syk

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Agonist-induced platelet activation triggers 'inside-out' signalling which activates α Ib- β 3, the most abundant integrin in platelet membranes. The engagement of activated α Ib- β 3 integrin by linking fibrinogen is necessary for platelet aggregation, and this induces subsequent outside-in signalling, which enhances platelet activation. Here we studied the involvement of Cbl during α Ib- β 3-integrin-mediated signal transduction. During thrombin-induced platelet activation, Cbl was tyrosine phosphorylated, and phosphoinositide 3-kinase (PI 3-kinase) activity measured in Cbl immunoprecipitates was increased. Both Cbl phosphorylation and its association with PI 3-kinase were dependent on α Ib- β 3 engagement by linking fibrinogen. The P256 and anti-LIBS6 (ligand-induced binding site 6) antibodies, which activate platelets directly through α Ib- β 3, induced

Cbl phosphorylation and increased the PI 3-kinase activity associated with Cbl. Both thrombin and antibodies to α Ib- β 3 induced association of Cbl with the tyrosine kinase, Syk. Experiments performed with inhibitors of tyrosine kinases indicated that both Src-family kinases and Syk contribute to phosphorylation of Cbl and its consequent association with PI 3-kinase. The results show that, following integrin α Ib- β 3 engagement, Cbl is tyrosine phosphorylated, recruits PI 3-kinase to this integrin signalling pathway and possibly enhances PI 3-kinase activity, downstream of Src-family tyrosine kinases and Syk activation.

Key words: fibrinogen binding, monoclonal antibodies, PI 3-kinase activity, platelet activation, thrombin.

INTRODUCTION

Human blood platelets are terminally differentiated and anucleated cells, the activation of which plays a key role in normal haemostasis. A number of agonists, including thrombin, collagen, thromboxane A_2 , ADP, noradrenaline (epinephrine) and von Willebrand factor, are able to stimulate platelets to different degrees. Depending on the agonist, platelet activation is accompanied by shape change, aggregation and release of granule contents [1,2]. Platelet activation involves an inside-out signalling, which activates the aggregation receptor, integrin α Ib- β 3 [also called glycoprotein IIb-IIIa (GPIIb-IIIa)]. Activated α Ib- β 3 links fibrinogen, which allows platelet aggregation and hence triggers an outside-in signalling. The latter event is necessary for full platelet spreading and aggregation, and effective haemostatic plug formation [3]. Outside-in signalling regulates the activation and tyrosine phosphorylation of intracellular proteins such as p125FAK (where FAK is focal adhesion kinase), p72Syk, Btk, Tec, SHIP [Src homology (SH2)-containing inositol 5'-phosphatase] and ERK2 (extracellular-signal-regulated kinase 2), and that of the β 3 subunit itself [3–7]. The tyrosine-phosphorylated β 3 subunit associates with the adaptor proteins Grb2 (growth-factor-receptor-bound protein 2) and SHC (Src-homology collagen) [7]. The tyrosine motif in the α Ib- β 3 cytoplasmic domain is necessary for the outside-in signalling, and indispensable for normal mouse haemostasis [8].

Cbl protein is the product of the *c-cbl* proto-oncogene, the cellular homologue of the *v-cbl* oncogene present in the Cas-NS-1 retrovirus which induces pre-B cell lymphomas and myeloid leukaemias [9,10]. Cbl is an adaptor protein that is present in a wide variety of cell systems, is phosphorylated on tyrosine and serine, is associated with a number of signalling proteins, and is

involved in cell proliferation following activation of a number of cell systems [11–14]. In a recent report [15], using the platelet-derived growth factor receptor β as a direct substrate, Cbl was shown to have ubiquitin ligase activity. For the latter activity, Cbl requires its intact SH2 and RING finger domains [15]. In platelets, the first reports on Cbl concerned its tyrosine phosphorylation during activation by thrombopoietin, and by convulxin or collagen [16,17]. We have also shown that phosphorylation of Cbl and its association with p85/phosphoinositide 3-kinase (PI 3-kinase) were markedly increased after Fc γ RIIa-mediated platelet activation [18].

In a variety of cells, integrin-mediated cell activation induces tyrosine phosphorylation of Cbl. Indeed, Cbl is the major tyrosine-phosphorylated CrkL-binding protein in the megakaryoblastic cell line MO7e after stimulation through the β 1 integrin [19]. In pre-B cells, Cbl is phosphorylated on tyrosine, and is associated with the p85 subunit of PI 3-kinase after ligation of the β 1 integrin [20]. The transformation of fibroblasts by oncogenic forms of Cbl induces constitutive activation of integrin-dependent signalling events, suggesting an important role for Cbl in this pathway [21]. In macrophages, tyrosine phosphorylation of Cbl is gradual and persistent during integrin-mediated cell activation, and allows the association of Cbl with active proteins (Src, Fgr, Lyn and PI 3-kinase) and the translocation of Cbl to the plasma membrane [22,23]. To our knowledge, the engagement of Cbl in platelet integrin signalling pathways has not yet been studied.

In the present work, we demonstrate that, downstream of α Ib- β 3 integrin engagement, there is tyrosine phosphorylation of Cbl and an increase in Cbl-associated PI 3-kinase activity. Cbl phosphorylation and the association of Cbl with PI 3-kinase were dependent on the binding of fibrinogen to α Ib- β 3 integrin

Abbreviations used: FAK, focal adhesion kinase; LIBS6, ligand-induced binding site 6; mAb, monoclonal antibody; NP40, Nonidet P40; PI 3-kinase: phosphoinositide (phosphatidylinositol) 3-kinase; RGDS, Arg-Gly-Asp-Ser peptide; SH, Src homology.

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after platelet activation by thrombin, but not after direct activation through α IIB- β 3 by monoclonal antibodies. α IIB- β 3 integrin engagement also led to the association of Cbl with the tyrosine kinase, Syk.

EXPERIMENTAL

Antibodies and reagents

Anti-Cbl polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The anti-Fc γ RII monoclonal antibody (mAb) IV.3 was from Medarex (West Lebanon, NH, U.S.A.). Anti-LIBS6 (ligand-induced binding site 6) and P256 activating mAbs were kindly donated by Professor M. H. Ginsberg (The Scripps Research Institute, La Jolla, CA, U.S.A.) and Dr N. Hogg (Imperial Cancer Research Fund, London, U.K.) respectively. Sheep anti-mouse horseradish-peroxidase-labelled antibody was from Amersham (Little Chalfont, Bucks., U.K.). Irrelevant mAbs and polyclonal antibodies were from Immunotech (Marseille, France). Anti-p85/PI 3-kinase anti-serum was from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Goat anti-rabbit horseradish peroxidase-labelled antibody was from Bio-Rad (Ivry Sur Seine, France). The anti-phosphotyrosine monoclonal antibodies PY20 and 4G10 were from Transduction Laboratories (Lexington, KY, U.S.A.) and Upstate Biotechnology respectively. Human thrombin was from Diagnostica-STAGO (Asnières, France). The Src-family kinase inhibitor PP2 was from Calbiochem (San Diego, CA, U.S.A.). Phosphatidylinositol, Protein A-Sepharose, leupeptin, aprotinin, piceatannol, PMSF, metrizamide and isopropyl β -D-thiogalactoside were from Sigma (Saint Quentin Fallavier, France).

Platelet preparation

Human platelets were isolated from fresh platelet concentrates obtained from healthy donors who had not taken aspirin for at least 1 week. The concentrates were centrifuged at room temperature for 15 min at 130 g, and then subjected to a washing process as described previously [24]. Briefly, platelets were isolated on a metrizamide gradient, collected and resuspended in 10 mM Hepes buffer, pH 7.4, containing 140 mM NaCl, 5 mM NaHCO₃, 0.5 mM MgCl₂, 3 mM KCl and 10 mM glucose. The platelet concentration was adjusted to 10⁹/ml for immunoprecipitation studies. CaCl₂ (1 mM) was added 10 min before platelet stimulation.

Platelet activation

Platelets were stimulated with P256 or anti-LIBS6 antibodies (10 μ g/ml) or with human thrombin (1 unit/ml) at 37 °C in an aggregometer (Coulter, Havertown, PA, U.S.A.) under constant stirring (1100 rev./min), in the presence or in the absence of Arg-Gly-Asp-Ser peptide (RGDS; 100 μ g/ml) to inhibit fibrinogen binding to α IIB- β 3 and platelet aggregation. RGDS was also tested at 400 μ g/ml, and gave the same results as at 100 μ g/ml. Fibrinogen (200 μ g/ml) was added to platelets activated by mAbs against α IIB- β 3. In order to inhibit the Fc receptor signalling pathway, platelets were preincubated for 1 min at 37 °C with mAb IV.3 (10 μ g/ml) before the addition of either P256 or anti-LIBS6 antibodies.

Immunoprecipitation and Western blot analysis

Immunoprecipitation studies and Western blot analysis were performed as described previously [18]. The membranes were stripped to remove the bound antibody by incubation in a

solution containing 2 % SDS, 62.5 mM Tris, pH 6.8, and 100 mM 2-mercaptoethanol for 40 min at 60 °C. After extensive washing, the membranes were re-probed. To control for the recovery of the immunoprecipitated Cbl protein, all membranes were re-probed with the anti-Cbl antibody.

Platelet cytoskeleton analysis

The Nonidet P40 (NP40)-insoluble fraction (cytoskeleton) was isolated as described previously [18], and washed three times with NP40-containing buffer (1 % NP40, 50 mM Tris, 50 mM NaCl, 5 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 mM EGTA, 1 mM Na₃VO₄ and 1 mM PMSF). The cytoskeletal fraction was solubilized in an SDS solution (10 % SDS and 5 mM EDTA), and 1 vol. of 4 $\times$ concentrated Laemmli sample buffer was added to 4 vol. of solubilized cytoskeletal fraction, with 5 % 2-mercaptoethanol. The samples were subjected to SDS/PAGE and Western blot analysis.

Measurement of PI 3-kinase activity

The measurement of PI 3-kinase activity was performed using immunoprecipitates obtained as described above, with additional extensive washing at 4 °C. Immunoprecipitates were washed twice in 20 mM Hepes containing 10 % glycerol, 1 % NP40, 150 mM NaCl and 1 mM vanadate; twice in PBS containing 1 % NP40 and 100 μ M vanadate; twice in 0.5 M LiCl/0.1 M Tris, pH 7.5; and twice in 0.1 M NaCl/1 mM EDTA/20 mM Tris, pH 7.5. The last wash was performed in a kinase buffer (20 mM Tris, pH 7.5, 100 mM NaCl and 0.5 mM EGTA) containing 0.2 mM adenosine to inhibit PI 4-kinase activity. The assay was performed in the kinase buffer by adding 10 μ g of sonicated phosphatidylinositol (resuspended in 10 mM Hepes and 1 mM EGTA, pH 7.5), 100 μ M ATP, 25 mM MgCl₂ and 10 μ Ci of [γ -³²P]ATP to the immunoprecipitates, in a final volume of 50 μ l. The reaction was stopped after incubation for 10 min at room temperature by the addition of 150 μ l of a stop solution containing chloroform/methanol/11.6 M HCl (50:100:1, by vol.). The lipids were extracted by the addition of 100 μ l of chloroform. The extract was washed with 200 μ l of methanol/1 M HCl (1:1, v/v). The lipids were resolved using chloroform/methanol/28 % ammonium hydroxide/water (86:76:10:14, by vol.) on silica gel TLC plates precoated with potassium oxalate. The reaction product migrated to the same position as that produced by using a recombinant purified PI 3-kinase (a gift from Professor L. C. Cantley, Beth Israel Hospital, Boston, MA, U.S.A.) with the phosphatidylinositol substrate. This reaction product ([γ -³²P]-phosphatidylinositol 3-phosphate) was visualized by autoradiography and measured by liquid scintillation counting.

RESULTS

Cbl is tyrosine phosphorylated during platelet activation through α IIB- β 3 integrin

In order to determine the involvement of Cbl in α IIB- β 3-integrin-mediated signal transduction, we first investigated the state of Cbl tyrosine phosphorylation. To specifically study the outside-in signalling, we used two mAbs that activate platelets directly via α IIB- β 3 integrin. The two mAbs, P256 and anti-LIBS6, were used in the presence of IV.3, an anti-Fc γ RII mAb which inhibits the binding of the IgG Fc domain to Fc γ RII.

Cbl was immunoprecipitated and analysed by Western blotting with anti-phosphotyrosine antibodies. In resting platelets, Cbl was not tyrosine phosphorylated. Cbl phosphorylation was observed after 1 min of platelet activation by P256 mAb, peaked

Figure 1 Kinetics of Cbl tyrosine phosphorylation in platelets activated through α IIB- β 3 integrin

Platelets were stimulated with P256 mAb (10 μ g/ml) for various times, as indicated (min). To avoid any interaction of the P256 Fc domain with Fc γ R1la, platelets were preincubated for 1 min with the specific anti-Fc receptor mAb, IV.3 (10 μ g/ml). After lysis with NP40 buffer, Cbl was immunoprecipitated (IP). After resolution of the immunoprecipitated proteins using SDS/10%-PAGE, proteins were transferred to nitrocellulose membranes and immunoblotted with the anti-phosphotyrosine (anti-PY) antibodies 4G10 plus PY20 (upper panel). The same membranes were re-probed with anti-Cbl antibody (lower panel). A control immunoprecipitation was performed with a non-immune antibody (NI). Results are representative of three experiments.

between 2 and 10 min, and decreased thereafter (Figure 1, upper panel). Similar results were obtained with the anti-LIBS6 mAb, except that there was no decrease in Cbl phosphorylation at 20 min (results not shown). The same nitrocellulose membrane re-probed with anti-Cbl antibody showed that increased and/or decreased Cbl phosphorylation was not due to a difference in the recovery of the protein (Figure 1, lower panel). The results indicated that Cbl was a substrate of tyrosine kinases downstream of α IIB- β 3 integrin engagement.

Effects of fibrinogen binding to α IIB- β 3 integrin and outside-in signalling on Cbl tyrosine phosphorylation

The major role of the α IIB- β 3 integrin is to mediate platelet aggregation by linking fibrinogen. We therefore investigated whether the Cbl phosphorylation that we have observed previously in thrombin-activated platelets [18] is a consequence of fibrinogen binding to α IIB- β 3. For this purpose, we studied platelet activation by thrombin in the presence or absence of RGDS, which inhibits fibrinogen binding to activated α IIB- β 3 integrin, and hence platelet aggregation and outside-in signalling. In the presence of RGDS, thrombin-induced Cbl tyrosine phosphorylation was totally inhibited (Figure 2a). In contrast, after activation by mAbs against α IIB- β 3 integrin (P256 or anti-LIBS6) in the presence of IV.3, Cbl phosphorylation was unmodified by the presence of RGDS for at least 10 min (Figures 2b and 2c), although platelet aggregation was totally abolished (results not shown). A small decrease in Cbl phosphorylation was observed, however, in the presence of RGDS after 20 min of platelet activation by anti-LIBS6 or P256 mAbs (Figures 2b and 2c). This suggests that fibrinogen binding contributes to Cbl phosphorylation at later times of platelet activation induced by mAbs against α IIB- β 3. In thrombin-activated platelets under non-stirred conditions, the profile of Cbl phosphorylation was similar to that obtained with stirring (results not shown). The latter result suggests that fibrinogen binding to α IIB- β 3 is required for Cbl phosphorylation, but that platelet aggregation is not. During thrombin receptor engagement, Cbl was involved in the

Figure 2 Role of α IIB- β 3 integrin engagement in thrombin-induced tyrosine phosphorylation of Cbl

Platelets were activated either by thrombin (1 unit/ml) (a), or by the anti- α IIB- β 3 mAbs (10 μ g/ml) anti-LIBS6 (b) or P256 (c) in the presence of IV.3 (10 μ g/ml), and in the presence or in the absence of RGDS (100 μ g/ml), for the indicated times (min). Platelets were then lysed by NP40-containing buffer and Cbl was immunoprecipitated (IP). The immunoprecipitates were subjected to SDS/PAGE, followed by anti-phosphotyrosine (anti-PY) immunoblotting. The same membranes were re-probed with anti-Cbl antibody. Results are representative of three experiments.

outside-in signalling (post-occupancy events) following fibrinogen binding to α IIB- β 3, but not in early events of the inside-out signalling which activates α IIB- β 3 integrin. The binding of the activating antibodies to α IIB- β 3 is, by itself, able to induce the outside-in signalling. Hence Cbl was phosphorylated independently of fibrinogen binding when platelet activation was mediated by mAbs to α IIB- β 3 integrin.

Src-family tyrosine kinases and Syk participate in Cbl phosphorylation in platelets

In order to investigate the involvement of tyrosine kinases in Cbl phosphorylation, we used a Src-family kinase inhibitor, PP2, and the Syk kinase inhibitor, piceatannol. In the concentration range 10–30 μ g/ml, piceatannol strongly inhibits Syk, but not Src-family kinases [25]. Our results show that, in thrombin-activated platelets (Figure 3a), Cbl phosphorylation was slightly decreased in the presence of 50 nM PP2, but was strongly inhibited by 5 μ g/ml piceatannol and was totally abolished by 20 μ g/ml piceatannol or by PP2 at 500 nM or 1 μ M. When platelets were activated by mAb P256 (Figure 3b), a low dose of piceatannol (5 μ g/ml) did not affect Cbl tyrosine phosphorylation. Cbl phosphorylation was decreased in the presence of 50 nM PP2, was strongly inhibited by 500 nM PP2 or 20 μ g/ml piceatannol, and was totally abolished by 1 μ M PP2. These data indicate that

Figure 3 Involvement of Syk and Src-family tyrosine kinases in Cbl phosphorylation

Platelets were incubated for 15 min at 37 °C with different concentrations of the inhibitors piceatannol (Pic) or PP2. Platelets were activated for 5 min by either thrombin (1 unit/ml) (a) or P256 mAb (10 µg/ml) (b). After cell lysis by NP40 buffer, Cbl was immunoprecipitated (IP). Cbl immunoprecipitates were subjected to SDS/PAGE and Western blot analysis using anti-phosphotyrosine (anti-PY) antibodies. The same membranes were then re-probed with anti-Cbl antibody. Results are representative of three experiments.

both Src-family and Syk kinases contribute to Cbl tyrosine phosphorylation in platelets activated through α IIb- β 3.

Association of Cbl with the actin cytoskeleton

Next we analysed the translocation of Cbl to the platelet actin cytoskeleton. In resting platelets, Cbl was present only in the NP40-soluble fraction, and was absent from the NP40-insoluble fraction corresponding to the actin cytoskeleton (Figures 4a and 4b). Following platelet activation by thrombin or mAbs to α IIb- β 3, the quantity of Cbl in the cytoskeletal fraction increased over time (Figures 4a and 4b, upper panels). This was accompanied by a parallel decrease in the amount of Cbl in the soluble fraction (Figures 4a and 4b, lower panels). Interestingly, RGDS did not modify the profile of Cbl association with the actin cytoskeleton in platelets activated by thrombin or mAbs (Figures 4a and 4b). In contrast, the tyrosine kinase inhibitors piceatannol and tyrphostin B56 partially inhibited the association of Cbl with the actin cytoskeleton induced by mAb P256 (Figure 4c). The tyrosine phosphatase inhibitor phenylarsine oxide increased Cbl translocation (Figure 4c). These results indicate that Cbl is translocated in the cytoskeleton during platelet activation, independently of fibrinogen binding and platelet aggregation, and this translocation depends in part on the activation of tyrosine kinases.

Cbl associates with PI 3-kinase following α IIb- β 3 integrin engagement

PI 3-kinase is involved in platelet signalling mediated by α IIb- β 3 integrin [26–29]. Since p85/PI 3-kinases are known to be regulated by the association of p85 with phosphotyrosine motifs on other proteins [30], we investigated whether Cbl could be a candidate protein to stimulate the PI 3-kinase activity following α IIb- β 3 integrin engagement and platelet aggregation. Our results showed that the p85 subunit of PI 3-kinase was co-immuno-

Figure 4 Translocation of Cbl to the actin cytoskeleton of activated platelets

Platelets were activated in the presence or absence of 100 µg/ml RGDS by 1 unit/ml thrombin (a) or 10 µg/ml IV.3 + P256 mAbs (b) for different times (min), as indicated. After cell lysis by NP40-containing buffer, the NP40-insoluble fraction (cytoskeleton) was separated from the soluble fraction. The samples from both fractions were subjected to SDS/PAGE and Western blot analysis by anti-Cbl antibody. The cytoskeleton of platelets activated by 10 µg/ml IV.3 + P256 mAbs in the presence of 20 µg/ml piceatannol, 100 µM tyrphostin B56 or 10 µM phenylarsine oxide (PAO) was separated and probed with anti-Cbl antibody (c). Results are representative of three experiments.

precipitated with Cbl between 2 and 10 min of platelet activation by thrombin (Figure 5a), although this association was low compared with that induced after Fc receptor engagement [18]. The Cbl–PI 3-kinase association was decreased at 20 min of platelet activation, and depended on fibrinogen binding to α IIb- β 3, since RGDS inhibited the transient Cbl–PI 3-kinase interaction (Figure 5a). We have also observed an association of Cbl with PI 3-kinase in thrombin-activated platelets under non-stirred conditions (results not shown). In platelets activated by

Figure 5 Association of Cbl with p85/PI 3-kinase in α IIb- β 3-mediated platelet activation

The nitrocellulose membranes corresponding to Figure 2 were re-probed with antibody against p85/PI 3-kinase. (a) Anti-Cbl immunoprecipitate (IP) from platelets activated by thrombin (1 unit/ml) in the presence or in the absence of RGDS. (b) Anti-Cbl immunoprecipitate from platelets activated by IV.3 + anti-LIBS6, in the presence or in the absence of RGDS. Results are representative of three experiments.

Figure 6 Measurement of PI 3-kinase activity associated with Cbl

Platelets were activated for 5 min by thrombin, IV.3 + P256 mAbs or IV.3 + anti-LIBS6 mAbs in the presence or in the absence of RGDS. Lysates were subjected to anti-Cbl immunoprecipitation. A control immunoprecipitation was performed using non-immune antibody. After extensive washing of the immunoprecipitates, the PI 3-kinase assay was performed. Data are means $\pm$ S.E.M. of three independent experiments.

the anti-LIBS6 mAb, the association between Cbl and PI 3-kinase was stable up to 20 min, and was unaffected by RGDS (Figure 5b). The P256 mAb induced a profile for the Cbl-PI 3-kinase association similar to that induced by anti-LIBS6 (results not shown).

Next we measured the PI 3-kinase activity in Cbl immunoprecipitates. In platelets activated by either thrombin or mAbs to α IIb- β 3, the PI 3-kinase activity in Cbl immunoprecipitates increased by 3–5 times over the basal activity observed in the resting state (Figure 6). In thrombin-activated platelets the PI 3-kinase activity associated with Cbl was strongly inhibited by the presence of RGDS, whereas this activity was not affected by RGDS in platelets activated by mAbs to α IIb- β 3 (IV.3 + anti-LIBS6 or IV.3 + P256) (Figure 6). Given the correlation observed between Cbl phosphorylation (Figure 2a) and its association with p85/PI 3-kinase (Figures 5 and 6), our results suggest that the association of Cbl with PI 3-kinase depends on Cbl tyrosine phosphorylation. In thrombin-activated platelets, the Cbl association with PI 3-kinase was due mainly to fibrinogen binding to α IIb- β 3 and the subsequent outside-in signalling, whereas in platelets activated by mAbs to α IIb- β 3 integrin it was independent of fibrinogen binding to α IIb- β 3.

To establish whether phosphorylation of Cbl is required for its association with PI 3-kinase, we studied the effects of the inhibitors PP2 and piceatannol on the Cbl-associated PI 3-kinase activity. The increased PI 3-kinase activity in Cbl immunoprecipitates observed after platelet activation by thrombin was inhibited by $67 \pm 14\%$ in the presence of 50 nM PP2 (Figure 7a, bar 4), and was totally inhibited by 500 nM PP2 or by 20 μ g/ml piceatannol (Figure 7a, bars 5 and 7). This activity was inhibited by $92 \pm 13\%$ in the presence of 5 μ g/ml piceatannol (Figure 7a, bar 6). In platelets activated by mAb P256 (Figure 7b), the increased PI 3-kinase activity associated with Cbl immunoprecipitates was inhibited by $68 \pm 13\%$ and $68 \pm 6\%$ by low concentrations of PP2 (50 nM) and of piceatannol (5 μ g/ml) respectively (Figure 7b, bars 4 and 6). This activity was inhibited by $85 \pm 10\%$ in the presence of 500 nM PP2 (bar 5), and was totally abolished by 20 μ g/ml piceatannol (bar 7). The decrease

Figure 7 Effects of inhibition of Src-family kinases and Syk on the association of PI 3-kinase with Cbl

Platelets were incubated with PP2 or piceatannol as in Figure 3. Activation was performed for 5 min either with thrombin (1 unit/ml) (a) or with IV.3 + P256 mAbs (10 μ g/ml) (b). Bars: 2, resting platelets; 3, activated platelets in the absence of inhibitors; 4–7, activated platelets in the presence of inhibitors: 50 nM PP2 (4), 500 nM PP2 (5), 5 μ g/ml piceatannol (6) or 20 μ g/ml piceatannol (7). After cell lysis by NP40-containing buffer, Cbl was immunoprecipitated (bars 2–7). A control immunoprecipitation was performed with non-immune antibody (bar 1). PI 3-kinase activity was measured in each immunoprecipitate. Data are means $\pm$ S.E.M. from three independent experiments. The insets show Western blots with anti-p85/PI 3-kinase performed on Cbl immunoprecipitates obtained using the same conditions.

in PI 3-kinase activity associated with Cbl corresponded to the decrease in the amount of p85/PI 3-kinase present in Cbl immunoprecipitates, as shown by Western blots (insets of Figures 7a and 7b). The results favour a role for tyrosine-phosphorylated Cbl in the positive regulation of PI 3-kinase activity, downstream of the activation of Src-family tyrosine kinases and Syk.

Association of Cbl with Syk in activated platelets

Cbl is known to negatively regulate Syk tyrosine kinase activity in mast cells and COS-7 cells [31,32]. We asked whether Cbl associates with Syk in platelets. For this purpose, we tested for the presence of Syk in Cbl immunoprecipitates. In resting platelets, Syk was hardly detectable in Cbl immunoprecipitates, whereas during platelet activation Cbl became associated with Syk. In thrombin-activated platelets, the Syk-Cbl association was observed at 2 min, reached a plateau between 5 and 10 min, and decreased thereafter, although it was still detectable at 20 min (Figure 8a). In the presence of RGDS, the thrombin-induced Cbl-Syk association was inhibited at 5 min and 10 min (although not totally), and was unchanged at 2 min and at 20 min (Figure 8a). Direct activation through α IIb- β 3 by mAbs

Figure 8 Association of Cbl with Syk in activated platelets

The nitrocellulose membranes corresponding to Figure 2 were re-probed with anti-Syk antibody. (a) Anti-Cbl immunoprecipitate (IP) from platelets activated by thrombin (1 unit/ml), in the presence or in the absence of RGDS. (b) Anti-Cbl immunoprecipitate from platelets activated by IV.3 + anti-LIBS6 mAbs, in the presence or in the absence of RGDS. Results are representative of three experiments.

Figure 9 Association of Syk with the β_3 subunit of $\alpha\text{IIb-}\beta_3$ integrin

Washed platelets were activated for 2 min by thrombin (1 unit/ml) in the presence or absence of RGDS (100 $\mu\text{g/ml}$), and lysed by NP40-containing buffer. $\alpha\text{IIb-}\beta_3$ was then immunoprecipitated (IP) from the NP40-soluble fraction, and a control immunoprecipitation was performed using non-immune antibody (NI). The samples were subjected to SDS/PAGE and Western blot analysis using anti-Syk and anti- β_3 antibodies. Results are representative of three experiments.

induced the association of Cbl with Syk, which peaked at 1–10 min and had decreased by 20 min (Figure 8b). In contrast with thrombin, however, the presence of RGDS did not affect the Cbl–Syk association. These results demonstrate the association of Cbl with Syk during platelet activation through $\alpha\text{IIb-}\beta_3$. This association was partly dependent on $\alpha\text{IIb-}\beta_3$ engagement in thrombin-activated platelets.

In order to determine if Syk associates with the β_3 subunit of $\alpha\text{IIb-}\beta_3$ integrin, we searched for its presence in the immunoprecipitates of $\alpha\text{IIb-}\beta_3$. With resting platelets, no association of Syk with $\alpha\text{IIb-}\beta_3$ was observed. After activation by thrombin, Syk became associated with $\alpha\text{IIb-}\beta_3$, and this association was partly inhibited by the presence of RGDS (Figure 9). Moreover, we immunoprecipitated the αIIb and β_3 subunits separately, and found that only the β_3 subunit was tyrosine phosphorylated and associated with Syk in thrombin-activated platelets (results not shown). These results suggest that Syk could initiate the signalling that occurs following the engagement of $\alpha\text{IIb-}\beta_3$ integrin.

DISCUSSION

The $\alpha\text{IIb-}\beta_3$ integrin is one of the most abundant molecules present at the surface of the platelet membrane. It binds several adhesive proteins, including fibrinogen, fibronectin, vitronectin and von Willebrand factor. The binding of such ligands induces

post-occupancy events that participate in platelet activation [3]. The present results help to define further the $\alpha\text{IIb-}\beta_3$ integrin pathway in platelets, and demonstrate the involvement of Cbl, PI 3-kinase and Syk in this outside-in signalling.

The involvement of Cbl in the signal transduction mediated by the platelet integrin $\alpha\text{IIb-}\beta_3$ was studied using mAbs that activate $\alpha\text{IIb-}\beta_3$ directly, and by using thrombin. In thrombin-activated platelets, phosphorylation of Cbl depended on fibrinogen binding. Indeed, in the presence of RGDS to inhibit fibrinogen binding to $\alpha\text{IIb-}\beta_3$ and platelet aggregation, thrombin-induced Cbl phosphorylation was totally inhibited. Previous reports described the regulation of protein tyrosine phosphorylation by $\alpha\text{IIb-}\beta_3$ in thrombin-activated platelets [3]. After platelet activation by different agonists, the outside-in signalling subsequent to fibrinogen binding to $\alpha\text{IIb-}\beta_3$ increases platelet activation through the phosphorylation of a number of proteins. Cbl appears to be one of these proteins. It is known that the anti-LIBS6 and P256 mAbs bypass the inside-out signalling by direct binding to $\alpha\text{IIb-}\beta_3$ integrin, which initiates the outside-in signalling [33]. We therefore used these mAbs to activate $\alpha\text{IIb-}\beta_3$ directly and to study this integrin signalling pathway, with a special focus on the involvement of Cbl. Under these conditions, Cbl was phosphorylated independently of fibrinogen binding to $\alpha\text{IIb-}\beta_3$.

Cbl was translocated to the cytoskeleton independently of fibrinogen binding, since RGDS did not affect this translocation during platelet activation by thrombin or by anti- $\alpha\text{IIb-}\beta_3$ mAbs. The amount of tyrosine-phosphorylated proteins observed in the cytoskeletal fractions from thrombin-activated platelets decreased in the presence of RGDS (results not shown). Although the phosphorylation of Cbl in the cytoskeleton of thrombin-activated platelets in the presence of RGDS was not analysed specifically, the phosphorylation of proteins of approx. 120 kDa decreased (results not shown), suggesting that Cbl translocation was not dependent on its own phosphorylation. Tyrosine kinase inhibitors, such as tyrphostin B56 and piceatannol, delayed the Cbl translocation, indicating that tyrosine kinases participate in cytoskeletal reorganization. Thus in activated, non-aggregated platelets, Cbl is translocated to the cytoskeleton in a similar manner to many signalling proteins such as Src, Fyn and Lyn [34–36]. The association of Cbl with the cytoskeleton during platelet activation could contribute to the reorganization of the actin cytoskeleton, as demonstrated recently in fibroblasts, in which Cbl regulates the formation of actin lamellae, lamellipodia and membrane ruffles [37].

With the aim of understanding the mechanism by which the adaptor protein Cbl could be involved in the outside-in signalling, we searched for an association of this protein with signalling enzymes involved in the $\alpha\text{IIb-}\beta_3$ pathway. The involvement of PI 3-kinase in the $\alpha\text{IIb-}\beta_3$ signalling pathway is now well established [26–29]. Our results showed that Cbl-associated PI 3-kinase activity was increased in activated platelets. The association of Cbl with PI 3-kinase was dependent on fibrinogen binding to $\alpha\text{IIb-}\beta_3$ in thrombin-activated platelets, but not in platelets activated by mAbs to $\alpha\text{IIb-}\beta_3$. Notably, during platelet activation through the Fc receptor, Cbl phosphorylation and its association with PI 3-kinase were independent of fibrinogen binding (results not shown). Thus we demonstrated that, in platelets, Cbl is involved in two signalling pathways: that of the Fc receptor [18], and the outside-in signalling through $\alpha\text{IIb-}\beta_3$ (the present study). Moreover, it has been shown that Cbl is tyrosine phosphorylated in platelets activated by thrombopoietin, convulxin and collagen, but the involvement of $\alpha\text{IIb-}\beta_3$ engagement in this phenomenon was not investigated in these reports [16,17]. In the present study, the association of Cbl with PI 3-kinase paralleled the level of Cbl

phosphorylation. Altogether, the data are in favour of positive regulation of PI 3-kinase activity by Cbl in platelets, as in other cell types [38,39]. PI 3-kinase plays a role in the maintenance of the active state of α IIb- β 3 during platelet activation by thrombin [27,40]. Kovacsics et al. [27] and our own results (A. Saci, F. Rendu and C. Bachelot-Loza, unpublished work) demonstrate that wortmannin (an inhibitor of PI 3-kinase activity) inhibits platelet aggregation induced by mAbs to α IIb- β 3. Zhang et al. [29] described the stimulation of a C2-domain-containing PI 3-kinase isoform downstream of the α IIb- β 3 integrin. This PI 3-kinase isoform has no p85 regulatory subunit that can associate with phosphotyrosine proteins, and its proline-rich sequences cannot associate with Cbl, since this latter protein does not contain an SH3 domain. Thus, in the present study, the PI 3-kinase activity measured in anti-Cbl immunoprecipitates was most probably that of p85/PI 3-kinase, i.e. the type I PI 3-kinase. Finally, considering our results and those of Zhang et al. [29], it appears that at least two types of PI 3-kinases are activated after α IIb- β 3 integrin engagement, and that intact PI 3-kinase activity is necessary for this integrin pathway.

Among other signalling partners of Cbl, Src and Syk are two important kinases in platelet activation. We were unable to detect an association between Cbl and Src. In contrast, we showed that Cbl was associated with Syk in activated platelets. In the presence of RGDS, the association of Cbl with Syk was partly inhibited in platelets activated by thrombin, but was unaffected during activation by P256 or anti-LIBS6 mAbs. Three different types of interaction could occur in the association of Cbl with Syk in platelets. First, the SH2 domain of Cbl (recently described in the N-terminal region of Cbl [41]) could associate with the phosphotyrosine residues of Syk. This proposal is supported by the observations that during activation by thrombin: (i) in the presence of RGDS, Cbl was associated with Syk, although Cbl was not phosphorylated, and (ii) Syk tyrosine phosphorylation was partly independent of fibrinogen binding [42]. Secondly, the phosphotyrosine residues of Cbl could associate with the Syk SH2 domain. Indeed, after 1 min of platelet activation by F(ab)'2 fragments of P256 mAbs, Syk is not phosphorylated [43], whereas Cbl is tyrosine phosphorylated and associated with Syk for up to 20 min (the present results). Also, during platelet activation by thrombin in the presence of RGDS, Cbl phosphorylation was inhibited and its association with Syk decreased at 5 and 10 min. In this regard, Cbl consensus sequences containing phosphotyrosine residues were proposed to associate with the C-terminal SH2 domain of Syk [44,45]. Thirdly, we cannot exclude the possibility that other proteins that interact with Syk and Cbl could mediate the Cbl-Syk association in a multimolecular complex. The Syk-Cbl association could contribute to the negative regulation of Syk activity by phosphorylated Cbl in platelets, as observed in mast cells and COS-7 cells [31,32].

Members of the Src family and Syk kinases are involved in the tyrosine phosphorylation of Cbl in osteoclasts, COS-7 cells, fibroblasts and macrophages [12]. Moreover, recent data from COS-7 cells indicate that Cbl phosphorylation occurs downstream of a novel activated Syk/Vav pathway [46]. In activated platelets, both piceatannol and PP2 inhibited Cbl phosphorylation and Cbl-associated PI 3-kinase activity. With a low concentration of PP2 (50 nM) the inhibition was partial, suggesting that Syk could compensate for the inhibited Src-family kinases. Similarly, a low dose of piceatannol was unable to significantly inhibit Cbl phosphorylation in platelets activated by anti- α IIb- β 3 mAbs. Low doses of piceatannol may only partly inhibit Syk activity, as shown previously in *ex vivo* experiments [25]. Src-family kinases could also compensate for the inhibition

of Syk. Finally, we cannot exclude the possibility that other kinases could be partly inhibited by piceatannol. For example, piceatannol inhibits FAK in an isolated system [47], but this inhibition has not been confirmed in a cell system. The stronger inhibitory effects of piceatannol on Cbl phosphorylation during platelet activation by thrombin than during activation by mAbs to α IIb- β 3 could be due to the involvement of Syk in the inside-out signalling. Indeed, during thrombin receptor engagement, inhibition of Syk decreases the activation of α IIb- β 3, and consequently decreases fibrinogen binding and inhibits the outside-in signalling [47]. Our data suggest that both Src-family tyrosine kinases and Syk participate in the phosphorylation of Cbl and hence contribute to the association of Cbl with PI 3-kinase, downstream of activated α IIb- β 3.

Other molecular interactions could occur downstream of activated α IIb- β 3 integrin. We found that Syk associated with Src after platelet activation by thrombin, and that this association was partly inhibited by RGDS (results not shown). In the same conditions, Syk associated with the β 3 subunit of α IIb- β 3 integrin, whereas we could not detect any association of Src with α IIb- β 3 (results not shown). Taken together, we propose the following sequential chain of events: engagement of platelet α IIb- β 3 activates Syk and Src-family kinases, which phosphorylate Cbl and promote the association of the latter with p85/PI 3-kinase, and hence PI 3-kinase activation by Cbl. This model is in keeping with a recent study in B cells which demonstrated that Cbl increases PI 3-kinase activity, dependent in part on its phosphorylation by activated Syk [39].

In conclusion, the present results demonstrate that Cbl is involved in platelet α IIb- β 3 signalling pathway by recruiting PI 3-kinase downstream of Src-family kinases and Syk. Hence Cbl could positively regulate PI 3-kinase activity. Further studies are required to determine how the α IIb- β 3-mediated outside-in signalling is necessary for full platelet spreading and aggregation, and effective haemostatic plug formation.

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