Fc Receptor-mediated Platelet Activation Is Dependent on Phosphatidylinositol 3-Kinase Activation and Involves p120cbl*

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Abdelatif Saci, Sabine Pain, Francine Rendu, and Christilla Bachelot-Loza

From INSERM U428, Faculté de Pharmacie, Université Paris-V, 75270 Paris, France

The platelet receptor for the Fc domain of IgGs (FcγRIIa) triggers intracellular signaling through protein tyrosine phosphorylations leading to platelet aggregation. In this study, we focused on the adaptor protein p120cbl (Cbl), which became tyrosine-phosphorylated after platelet activation induced by antibodies. Cbl phosphorylation was dependent on Fc receptor engagement. An association of Cbl with the p85 subunit of phosphatidylinositol 3-kinase (PI 3-K) occurred in parallel with Cbl tyrosine phosphorylation. We showed by in vitro experiments that Cbl/p85 association was mediated by the Src homology 3 domain of p85/PI 3-K and the proline-rich region of Cbl. Inhibition of PI 3-K activity by wortmannin led to the blockade of both platelet aggregation and serotonin release mediated by FcγRIIa engagement, whereas it only partly inhibited those induced by thrombin. Thus, PI 3-K may play a crucial role in the initiation of platelet responses after FcγRIIa engagement. Our results suggest that Cbl is involved in platelet signal transduction by the recruitment of PI 3-K to the FcγRIIa pathway, possibly by increasing PI 3-K activity.

The Cbl protein is the product of the c-b blastogenic oncogene, the cellular homologue of the v-cbl oncogene present in the Caelus-1 retrovirus, which induces pre-B cell lymphomas and myeloid leukemias (1, 2). Cbl is found in a wide range of hematopoietic cell lineages and some nonhematopoietic tissues such as lung, brain, and testis (3). A deletion in the c-b region (62% of the C-terminal domain) involving functional domains, such as the leucine zipper motif and proline-rich region, converts this protooncogene into the transforming one (4). Unlike the product of v-cbl localized in both the cytoplasm and the nucleus, the c-b product (p120cbl) is exclusively cytoplasmic (5). A tumorigenic form of Cbl was detected in the 70Z/3 pre-B cell lymphoma, in which Cbl tyrosine phosphorylation is increased as a result of a deletion of 17 amino acids in the Cbl sequence (6). Cbl is also heavily tyrosine-phosphorylated in v-src-transformed hematopoietic cells (7).

Cbl becomes tyrosine-phosphorylated after cell stimulation through a wide range of receptors including B and T cell receptors (8–12), various growth factor receptors (13–21), integrins (22–24), and receptors for the Fc domain of IgGs (21, 25, 26). The primary structure of Cbl shows no homology with any catalytic domain but contains a number of tyrosine residues that can be phosphorylated and a proline-rich region (2). Cbl has been shown to bind to a number of signaling proteins, such as tyrosine kinases Src, Lyn, Lyn, Lck, Blk, Syk, the lipid kinase phosphatidylinositol 3-kinase (PI 3-K) (1), phospholipase Cy, and the adaptor proteins Grb2 and Vav (9–11, 22, 27–33). Cbl phosphorylation on serine residues has also been reported in phorbol ester-activated T cells, allowing its interaction with 14.3.3 protein (34). Finally, Cbl contains a phosphotyrosine binding domain in the N-terminal region that directly binds to phosphorylated ZAP-70 in activated T cells (35).

A regulator activity has recently been described for Cbl when overexpressed in mast cells, in which it regulates p72* activity (36). Cbl is also proposed to regulate the T cell receptor-mediated Ras pathway activation via its association with Grb2 in T cells (37). In interleukin 4-treated B cells, Cbl is tyrosine-phosphorylated and associated with p85/PI 3-K, and overexpression of Cbl enhances PI 3-K activity, mitogenic activity, and cell survival (38). Taken together, the data suggest a role for Cbl in multiple signaling pathways of different cell types.

In human platelets, Cbl has been identified and shown to be co-constitutively associated with the Grb2 adaptor protein and tyrosine-phosphorylated after thrombopoietin activation (27). Thus, Cbl seems to be implicated in signal transduction after thrombopoietin binding to c-mpl. However, the role of Cbl or its possible involvement in platelet signal transduction mediated by other receptors has not yet been documented. Platelet activation is mediated by a wide variety of agonists, including thrombin, thromboxane A2, ADP, and adhesion molecules such as von Willebrand factor and collagen. Some antibodies directed against antigens on the platelet membrane (e.g., the tetraspanin CD9, glycoprotein IV, and the integrins α5β3) are also able to induce platelet activation. In most cases, the activation induced by IgGs is dependent on the binding of their Fc domain on the specific receptor, FcγRIIa (39).

In the present study, we have investigated the involvement of Cbl in platelet signaling after FcγRIIa engagement. Two models of platelet activation involving the Fc receptor were used: the cross-linking of FcγRIIa and bridging of the CD9 antigen with FcγRIIa by an activating monoclonal antibody (mAb) (anti-CD9, Syb). Our results demonstrate that after FcγRIIa engagement, Cbl was heavily tyrosine-phosphorylated. In parallel with Cbl phosphorylation, we found that Cbl was associated with p85/PI 3-K. Moreover, the use of wortmannin, an inhibitor of PI 3-K, abolished platelet aggregation and release induced by antibodies, underlining the crucial role of PI 3-K during immunological activation. The results suggest an

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To whom correspondence should be addressed: INSERM U428, Faculté de Pharmacie, 4 ave. de l’Observatoire, F-75006, Paris, France. Tel.: 33-1-53-73-96-19; Fax: 33-1-44-07-17-72; E-mail: bachelot@pharmacie.univ-paris5.fr.

The abbreviations used are: PI 3-K, phosphatidylinositol 3-kinase; FcγRIIa, platelet receptor for the Fc domain of IgGs; mAb, monoclonal antibody; RAM, rabbit polyclonal F(ab)2anti-mAb; GST, glutathione S-transferase; IP, immunoprecipitation; PAGE, polyacrylamide gel electrophoresis; SH, Src homology; PY, phosphotyrosine.

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important role for Cbl in FcRIIa-mediated platelet activation, possibly through the regulation of PI 3-K activity.

EXPERIMENTAL PROCEDURES

Reagents—Anti-Cbl polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Syb-1, a monoclonal IgG anti-CD9 (40), was kindly provided by Dr. C. Bouchex (INSERM U268). Anti-FcγRIIa mAb IV.3 was from MEDAREX Inc. (West Lebanon, NH). Irrelevant monoclonal and polyclonal antibodies and rabbit polyclonal F(ab)_2 anti-mAb (RAM) were from Immunotech (Marseille, France). Anti-p85/PI 3-K antisera was from Upstate Biotechnology Inc. (Lake Placid, NY). Sheep anti-mAb horseradish peroxidase-labeled antibody was from Amersham Pharmacia Biotech. Goat anti-rabbit horseradish peroxidase-labeled antibody was from Bio-Rad. Anti-phosphotyrosine monoclonal antibodies PY20 and 4G10 were from Transduction Laboratories (Lexington, KY) and Upstate Biotechnology, respectively. Human thrombin was from Diagnostica-Stago (Asnière, France). Metrizamide was from Eurobio (Les Ulis, France), and protein A-Sepharose, leupeptin, aprotonin, phenylmethylsulfonyl fluoride, Wortmannin, and isopropyl-J-thiogalactopyranoside were from Sigma. Bacteria expressing p85/PI 3-K glutathione S-transferase (GST) fusion proteins were a kind gift from Prof. L. Cantley (Beth Israel Hospital, Boston, MA), and Dr. S. Fischer (INSERM U363, Paris, France).

Platelet Preparation—Human platelets were isolated from fresh platelet concentrate taken from healthy donors who did not taken aspirin for at least 1 week. The concentrates were centrifuged at room temperature for 15 min at 130 × g to eliminate other cell types and then subjected to a washing process as described previously (41). 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 NaHCO_3, 0.5 mM MgCl_2, 3 mM KCl, and 10 mM glucose. Platelet concentration was adjusted to 10^9/ml for immunoprecipitation studies or to 5.10^8/ml for studying the total platelet lysates. CaCl_2 (1 mM) was added 10 min before platelet stimulation.

Platelet Activation, Aggregation, and Release—Platelets were stimulated with either 10 μg/ml of antibodies or 1 unit/ml human thrombin at 37 °C in an agitometer (Coulter, Havertown, PA) with constant stirring (1100 rpm). For Fc receptor pathway inhibition, platelets were preincubated for 1 min at 37 °C with IV.3 (10 μg/ml) before addition of Syb antibody (40) or for Fc receptor cross-linking, platelets were preincubated for 1 min with IV.3 (10 μg/ml) and then stimulated by addition of RAM-F(ab)_2 (80 μg/ml) for various periods. To study the total platelet proteins, the reactions were stopped by addition of 25% (v/v) of a buffer containing 10% SDS and 5 mM EDTA, and the samples were transferred to ice for complete lysis. After 30 min, 25% (v/v) of a 4 × concentrated Laemmli’s buffer and 5% of 2-mercaptoethanol were added, and the samples were subjected to Western blot analysis.

To study the platelet aggregation and release, the platelet-rich plasma was adjusted to 5 × 10^9 platelets/ml and incubated with 0.6 μM 125I-labeled serotonin (Amersham Pharmacia Biotech) for 30 min at room temperature, and then the platelets were isolated as described above. Immunoprecipitation—For immunoprecipitation (IP) studies, platelet stimulation was performed with the addition of one-third volume of cold 3 × concentrated Nonidet P-40 lysis buffer containing 3% (v/v)Nonidet P-40, 150 mM Tris, 450 mM NaCl, 15 μg/ml leupeptin, 15 μg/ml aprotonin, 5 mM EDTA, 3 mM NaVO_3, and 3 mM phenylmethylsulfonyl fluoride. The platelets were incubated for 30 min at 0 °C before platelet activation, and the aggregation was determined by measuring changes in light transmission through a stirred volume of platelets at 37 °C in aggregometer. The aggregation was monitored for 5 min, and the reaction was stopped by transfer into 0.2 volume of ice-cold 0.1 M EDTA and immediate centrifugation for 1 min at 12,000 × g. The [125I]serotonin was measured in the supernatant by liquid scintillation counting. Release was expressed as % of [125I]serotonin liberated compared with the total unstimulated platelet content.

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For the preclerking experiments, antibody concentrations used for protein immunodepletion were raised to 5 μg/ml. The Nonidet P-40-soluble fraction was incubated with anti-Cbl, anti-phosphotyrosine (4G10), or control antibodies for 2 h, followed by addition of 40 μl of protein A-Sepharose (50% slurry). This step was reproduced after removing the immunocomplexes. Before addition of each antibody for IP, an aliquot (50 μl) of the platelet lysate was conserved in Laemmli’s buffer at −20 °C. Both immunoprecipitates and aliquot samples were subjected to Western blot analysis.

In some experiments, the membranes were stripped; the bound antibody was removed by incubation in buffer 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 membrane was reprobed with another antibody as described above.

GST Fusion Protein Studies—Cultures of bacteria expressing GST or GST fusion proteins (GST-SH3-(p85/PI 3-K), GST-(N- or C-terminal)-SH2-(p85/PI 3-K), and GST-p85 (full p85/PI 3-K)) were grown, and GST fusion proteins were isolated as described previously (42, 43). GST or GST fusion proteins bound to glutathione-Sepharose 4B were incubated with platelet lysates (Nonidet P-40-soluble fraction) for 2 h. After brief centrifugation the precipitates were washed and treated as described for the IP studies.

The GST fusion proteins were subjected to a competition study. In this case, the GST-p85 and GST-SH3 were preincubated 30 min with the proline-rich peptides corresponding to residues 82–96 and 300–314 of p85, which were previously shown to bind to the SH3 domain of the p85 itself (43), using an unrelated peptide as control (GSGVVRING-GRD). Precipitations were performed at 4 °C for 30 min with constant stirring, added to platelet lysates, and treated as described above.

RESULTS

Cbl Tyrosine Phosphorylation during Platelet Activation—To examine whether activation of platelets through FcRIIa involved Cbl, we activated platelets by Fc receptor cross-linking, using IV.3 (anti-Fc receptor) in the presence of RAM-F(ab)_2 or by an anti-CD9 antibody (Syb) known to induce platelet activation through an FcRIIa-dependent pathway. We also compared these results with those observed after thrombin stimulation.

Platelet activation mediated through FcγRIIa or the thrombin receptor induced an increase in the level of phosphotyrosine proteins (PYs). We focused our attention on a band at ~120 kDa, which was tyrosine-phosphorylated after 2 min of platelet activation (Fig. 1, upper panel). To see whether this band corresponded to p120 Cbl, the nitrocellulose membrane was reprobed with anti-Cbl antibody. This experiment confirmed the presence of Cbl in platelets, which is localized within the 120-kDa band (Fig. 1, lower panel). This result was further confirmed by preclerking experiments. Lysates of Syb-activated platelets were subjected twice to anti-PY or anti-Cbl immunoprecipitations. Many PYs were absent after immunodepletion of total PY, including the tyrosine-phosphorylated 120-kDa band (Fig. 2a, upper panel, lanes 4 and 6). A fraction of Cbl remained in the total lysate subjected to anti-PY immunoprecipitation, which probably corresponds to the phosphophorylated-Cbl (Fig. 2a, lower panel, lanes 4 and 6). In platelet lysates depleted of Cbl, the tyrosine-phosphorylated band at ~120 kDa remained unchanged (Fig. 2a, upper panel, lanes 2 and 5), indicating the presence of additional proteins in the 120-kDa band. Anti-Cbl immunoblotting of the same membrane confirmed the total depletion of Cbl in the lysate (Fig. 2a, lower panel, lane 5). Furthermore, Cbl was present in the anti-PY immunoprecipitated...
tates of Syb-activated platelets (Fig. 2b, lanes 3 and 6). Altogether, these data confirmed the presence of Cbl within the 120-kDa band and indicated that in Syb-activated platelets, the 120-kDa phosphotyrosine band corresponds to several PYs including Cbl.

To determine the levels of Cbl tyrosine phosphorylation induced by the former agonists, Cbl was immunoprecipitated from lysates of resting and activated platelets. As shown in Fig. 3, upper panel, Cbl was not significantly tyrosine-phosphorylated in resting platelets. By contrast, a high level of Cbl tyrosine phosphorylation was observed after FcγRIIa cross-linking. Cbl was also strongly tyrosine-phosphorylated in Syb-activated platelets, although to a lesser level than after FcγRIIa cross-linking. Cbl was only minimally phosphorylated in thrombin-activated platelets (Fig. 3b). Densitometer scanning of the autoradiographs of five experiments confirmed this difference (Fig. 3b). Reprobing the membrane with anti-Cbl antibody (Fig. 3, lower panel) confirmed that the increase in tyrosine phosphorylation of Cbl was not the consequence of a change in the recovery of the protein. The addition of IV.3 alone did not induce any tyrosine phosphorylation. Preincubation of IV.3 to block the binding of Syb Fc domain to FcγRIIa totally inhibited Syb-induced tyrosine phosphorylation of Cbl. The results demonstrate that Cbl was heavily tyrosine-phosphorylated after FcγRIIa engagement and suggest a Cbl involvement in FcγRIIa-mediated platelet signaling.

To verify that the different levels of Cbl tyrosine phosphorylation (depending on the agonist used) were not attributable to different kinetics and to determine when Cbl phosphorylation occurred, we studied the time course of Cbl tyrosine phosphorylation during platelet stimulation. Strong Cbl tyrosine phosphorylation was already reached at 30 s after platelet activation induced by FcγRIIa cross-linking, with a plateau obtained between 1 and 2 min and a decrease to lower levels thereafter (Fig. 4, upper panel). Syb-induced Cbl tyrosine phosphorylation kinetics was similar to that observed after FcγRIIa

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**Fig. 1. Identification of Cbl among tyrosine-phosphorylated proteins during platelet activation.** Washed platelets were left untreated or were treated by different agonists for 2 min under constant stirring. At the end of the stimulation period, samples were solubilized and resolved on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with a mixture of 4G10 and PY20 anti-phosphotyrosine antibodies (upper panel). Lower panel, same nitrocellulose membrane stripped and immunoblotted with anti-Cbl antibody. IV.3, anti-FcγRII antibody (10 μg/ml); RAM, F(ab′)2 rabbit anti-mouse antibody (80 μg/ml); Syb, anti-CD9 antibody (10 μg/ml); thrombin (1 unit/ml). Results are representative of five experiments.

**Fig. 2. Depletion of Cbl and phosphotyrosine proteins in platelet lysates.** Washed platelets were activated with Syb antibody (10 μg/ml). After 2 min of stimulation, the cells were lysed with Nonidet P-40 buffer, and the lysates were immunoprecipitated twice with either anti-Cbl antibody or anti-PY antibodies (5 μg/ml). a, aliquots of each lysate were collected and conserved at –20 °C before and after each immunoprecipitation. Whole platelet lysates were subjected to SDS-PAGE and immunoblotting with anti-PY (upper panel) or anti-Cbl antibodies (lower panel). Platelet lysates: lane 1, before any IP; lane 2, after the first IP with anti-Cbl antibody; lane 3, after control IP with nonimmune antibody; lane 4, after control IP with anti-PY; lane 5, after the second IP with anti-Cbl; lane 6, after the second IP with anti-PY. b, immunoprecipitates of the first and second control IPs (lanes 1 and 4), first and second anti-Cbl IPs (lanes 2 and 5), and first and second anti-PY IPs (lanes 3 and 6) were solubilized and resolved on SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti-Cbl. Results are representative of two experiments.
cross-linking (Fig. 4, middle panel), with or without a lag depending on the donor used. Indeed, the FcγRIIa His-Arg-131 polymorphism has been shown to play a crucial role in the ability of FcγRIIa to bind the Fc domain of mAb-IgG1 and, consequently, in the cell activation induced by these antibodies (44). We and others have shown that platelets from homozy- gous His donors respond more slowly than platelets from homozygous Arg donors to anti-CD9 antibodies, whereas the lag phase of platelets from heterozygous donors is intermediate (41, 44). Thrombin induced a weak and slow Cbl tyrosine phosphorylation, which peaked between 2 and 5 min after platelet stimulation and decreased thereafter (Fig. 4, lower panel). The data suggest that Cbl was tyrosine-phosphorylated in the early stages of platelet activation and could participate in the first events triggered by Fc receptor engagement. During thrombin activation, Cbl would be involved to a lesser extent and in later stages after platelet activation.

Cbl Association with p85/PI 3-K in Activated Platelets—As an adaptor protein, tyrosine-phosphorylated Cbl was shown to associate with various signaling proteins. Among them, Cbl was reported in various cells to associate with PI 3-K (9, 28, 29, 45) and to enhance PI 3-K activity (27, 38). Because PI 3-K plays an important role in platelet function (46), we searched for an association between Cbl and PI 3-K in platelets. Cbl was immunoprecipitated from resting and stimulated platelets and samples were analyzed with an anti-p85/PI 3-K antibody. In resting platelets, p85/PI 3-K was hardly detectable in the Cbl immunoprecipitates (Fig. 5). In contrast, p85/PI 3-K co-immunoprecipitated with Cbl in platelets activated through the Fc receptor. In thrombin-activated platelets, Cbl/PI 3-K association was dependent on Cbl tyrosine phosphorylation.

To determine which domain of p85 was involved in Cbl/PI 3-K association, we studied Cbl binding to bacterial GST fusion proteins corresponding to full p85 (GST-P85), the SH3 domain of p85 (GST-SH3), or the N- and C-terminal SH2 domains of p85. Incubation of the different GST fusion proteins with platelet lysates showed that none of the two p85 SH2 domains (C- and N-terminal) bound Cbl (Fig. 6a). By contrast, an association of p85 (full) or p85 SH3 domain with Cbl was observed in both resting and activated platelet lysates. These data suggested an interaction between the p85 SH3 domain and the Cbl proline-rich region.

To determine whether Cbl/PI 3-K association was mediated by the p85 SH3 domain and the Cbl proline-rich region, we used competitive proline-rich peptides derived from p85 that were previously shown to bind the SH3 domain of p85 itself (43). The two competitive peptides strongly abolished Cbl association with GST-SH3 (p85) and GST-P85 (full). The inhibition was total when the two peptides were added together (Fig. 6b), indicating that, in vitro, the p85 SH3 domain mediates Cbl/p85 association through its interaction with the Cbl proline-rich region.

Inhibition of PI 3-K Activity Abolished the Platelet Responses after FcγRIIa Engagement—To determine whether PI 3-K plays a role in platelet activation mediated by the Fc receptor, we studied the effect of wortmannin (50 nM), an inhibitor of PI 3-K activity, on platelet aggregation and serotonin release. Platelet aggregation induced by FcγRIIa cross-linking or by Syb was strongly inhibited by wortmannin (100 and 88%, respectively; Fig. 7). By contrast, platelet aggregation induced by thrombin was poorly inhibited in the presence of wortmannin and became reversible. Serotonin release induced by FcγRIIa engagement was greatly inhibited by preincubation of platelets with wortmannin. Indeed, 85% inhibition of serotonin release was observed in platelets activated by cross-linking, and 70% inhibition was observed after activation by Syb. Wortmannin had no significant effect on thrombin-induced serotonin release (Fig. 7). The data suggest a key role for PI 3-K in Fc receptor-mediated platelet activation. If tyrosine-phosphorylated Cbl enhances PI 3-K activity, Cbl would also play an important role in platelet activation.
Cbl and PI 3-K Involvement in FcγRIIA-mediated Platelet Activation

FIG. 5. Cbl association with p85/PI 3-K. The nitrocellulose membranes corresponding to Figs. 3 and 4 were reprobed with anti-p85/PI 3-K antibody. a, IP anti-Cbl from platelets incubated 2 min with IV.3 (10 μg/ml), IV.3 plus Fab’2 RAM (80 μg/ml), Syb (10 μg/ml), or thrombin (1 unit/ml). b, IP anti-Cbl from platelets activated for 30 s to 10 min by IV.3 plus RAM, Syb, or thrombin. Results are representative of four experiments.

DISCUSSION

The adaptor protein Cbl has been identified in a variety of cells, including platelets, but its involvement in platelet signaling remains uncharacterized. The present work was devoted to studying the involvement of Cbl in signal transduction after platelet activation induced by FcγRIIA cross-linking or Syb antibody (anti-CD9), which activates platelets via FcγRIIa. We demonstrated strong and rapid tyrosine phosphorylation of Cbl in platelets activated through FcγRIIa. In addition, we showed that after platelet activation, p85/PI 3-K association with Cbl correlates with the intensity of Cbl tyrosine phosphorylation. Furthermore, we showed that the PI 3-K inhibitor wortmannin abolished antibody-mediated platelet responses, indicating a crucial role for PI 3-K in antibody-induced platelet activation.

Cbl was not significantly tyrosine-phosphorylated in resting platelets, but it became phosphorylated during platelet activation depending on the agonist used. After activation by FcγRIIa cross-linking, Cbl was strongly and rapidly tyrosine-phosphorylated. To a lesser extent, Syb induced a similar Cbl phosphorylation to that obtained after FcγRIIa cross-linking. The former difference in the Cbl phosphorylation was probably attributable to distinct modes of platelet activation induced by Syb and FcγRIIa cross-linking, as suggested by others (47). That specific binding of Syb antibody to its antigen (CD9), in the presence of IV.3 (anti-FcγRI), did not induce Cbl phosphorylation indicates that Cbl tyrosine phosphorylation occurred after FcγRIIa engagement. These results suggest that, unlike thrombin, which induced a faint and slow Cbl tyrosine phosphorylation, Fc receptor engagement strongly involves Cbl at the first steps of platelet signaling. Another protein involved in the first steps of FcγRIIIA-mediated signal transduction is the tyrosine kinase Syk (48). The latter could be a potential candidate to phosphorylate Cbl in platelets, because it was previously demonstrated to participate in Cbl phosphorylation in activated T cells (49). We could not, however, detect any Cbl association with Syk after platelet activation. Cbl tyrosine phosphorylation was transient, suggesting an action of tyrosine phosphatase(s) on phosphorylated Cbl. This is supported by the fact that in the presence of the protein tyrosine phosphatase inhibitor phenylarsine oxide, Cbl phosphorylation remained stable for up to 10 min of platelet activation (data not shown).

Because Cbl association with p85/PI 3-K has been suggested to increase PI 3-K activity in a number of cell systems (14, 27, 38), we examined the association of Cbl with PI 3-K in platelets. We found that Cbl/PI 3-K association was negligible in resting and thrombin-activated platelets. In contrast, Cbl was strongly associated with PI 3-K after FcγRIIA-mediated platelet activation. In *in vitro* experiments, using GST fusion proteins, we did not find any association between the N-SH2 or C-SH2 domains of p85 and Cbl in resting or Syb-activated platelets. In contrast, full p85 and the p85 SH3 domain precipitated Cbl from resting and Syb-activated platelet lysates. It is thus most likely that Cbl and p85 associate via the SH3 domain of p85 and the proline-rich region of Cbl.

We could not exclude, however, that *in vivo* Cbl/PI 3-K association may require tyrosine phosphorylation of Cbl. Indeed, our experiments favor a relationship between these two events. In NH2 cells, constitutive Cbl/PI 3-K association is mediated by the Cbl proline-rich region and the p85 SH3 domain, whereas increased Cbl/p85 association was proposed to occur through both the p85 SH2 and SH3 domains after cell activation and Cbl tyrosine phosphorylation (27). In fact, both SH2 and SH3 domains of p85 interact with Cbl in other cells (19, 22, 33, 50). Interestingly, Solttoff and Cantley (19) suggested that engagement of the p85 SH2 domain exposes the SH3 domain, which can then further interact with Cbl and increase the affinity of p85 for Cbl. The authors proposed that Cbl could act as an adaptor protein that recruits PI 3-K in the epidermal growth factor-mediated activation of PC12 cells (19). Moreover, Cbl has a Tyr-X-X-Met motif, which could associate with a p85 SH2 domain if phosphorylated on tyrosine (51). Alternatively, the tyrosine phosphorylation of Cbl could be necessary for its relocalization near PI 3-K. In that respect, Tanaka et al. (25) showed that in epidermal growth factor-activated macrophages and fibroblasts, Cbl tyrosine phosphorylation may be accompanied by its subcellular translocation. A last hypothesis could be that in resting platelets, the Cbl proline-rich region may not be in a conformation that allows its association with the p85 SH3 domain. After platelet activation and Cbl phosphorylation, a conformational change in Cbl would render possible the association between the two proteins. By analogy, in stimulated fibroblasts, a phosphotyrosine-dependent conformational change of Cbl was proposed to transiently expose the Cbl N-terminal region, permitting interaction with platelet-derived growth factor receptor α (52).

To determine whether Cbl tyrosine phosphorylation and association with PI 3-K occurred before PI 3-K activation, we used wortmannin to inhibit PI 3-K activity. We found that wortmannin had no effect on Cbl phosphorylation or on Cbl/PI 3-K association induced by different agonists, which indicates that the two events occurred upstream of the lipid kinase activation (data not shown). However, a noncovalent association between PI 3-K and FcγRIIa has been previously shown (53). Therefore, tyrosine-phosphorylated Cbl would play a role in FcγRIIa-mediated platelet signaling by linking PI 3-K with the Fc receptor pathway, possibly by enhancing PI 3-K activity.

That the level of Cbl/p85 association was stronger after Fc receptor engagement than after thrombin addition suggests a differential role for PI 3-K in the signaling induced by the two types of platelet activation. In the presence of wortmannin, platelet aggregation mediated through FcγRIIa was abolished. In contrast, and as previously demonstrated with thrombin receptor activating peptide (54, 55), platelet aggregation induced by thrombin was only partially inhibited and became reversible. These data indicate that PI 3-K participates in the control of platelet aggregation, especially that which occurs after Fc receptor engagement. The crucial role of PI 3-K in FcγRIIa-mediated platelet activation was also confirmed by the demonstration that wortmannin strongly inhibited antibody-
induced serotonin release from dense granules but only weakly inhibited serotonin release induced by thrombin. These results demonstrated that in platelets, PI 3-K activation was required to initiate platelet responses after FcγRIIa engagement. Thus, if Cbl increases PI 3-K activity as previously proposed, Cbl could also play a crucial role in platelet activation mediated through FcγRIIa.

In conclusion, Cbl was strongly tyrosine-phosphorylated during FcγRIIa-mediated platelet activation, and levels of Cbl tyrosine phosphorylation paralleled levels of Cbl/PD PI 3-K association. Because PI 3-K activity appeared crucial in platelet responses dependent on FcγRIIa engagement, we suggest that Cbl participates in signal transduction mediated through the Fc receptor by enhancing PI 3-K activity. Thus, Cbl could be one of the key adaptor and regulator proteins in this system.

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