Cbl-b Is a Novel Physiologic Regulator of Glycoprotein VI-dependent Platelet Activation

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Cbl-b, a member of the Cbl family of E3 ubiquitin ligases, plays an important role in the activation of lymphocytes. However, its function in platelets remains unknown. We show that Cbl-b is expressed in human platelets along with c-Cbl, but in contrast to c-Cbl, it is not tyrosine-phosphorylated upon glycoprotein VI (GPVI) stimulation. Cbl-b, unlike c-Cbl, is not required for Syk ubiquitylation downstream of GPVI activation. Phospholipase Cγ2 (PLCγ2) and Bruton’s tyrosine kinase (BTK) are constitutively associated with Cbl-b. Cbl-b-deficient (Cbl-b−/−) platelets display an inhibition in the concentration-response curve for GPVI-specific agonist-induced aggregation, secretion, and Ca2+ mobilization. A parallel inhibition is found for activation of PLCγ2 and BTK. However, Syk activation is not affected by the absence of Cbl-b, indicating that Cbl-b acts downstream of Syk but upstream of BTK and PLCγ2. When Cbl-b−/− mice were tested in the ferric chloride thrombosis model, occlusion time was increased and clot stability was reduced compared with wild type controls. These data indicate that Cbl-b plays a positive modulatory role in GPVI-dependent platelet signaling, which translates to an important regulatory role in hemostasis and thrombosis in vivo.

Platelet activation by newly exposed basement membrane collagen is a key initial step in both hemostasis and thrombosis (1). The complex of glycoprotein VI (GPVI) and the Fc receptor γ chain is primarily responsible for activation of platelets by collagen (2). This receptor acts via a PLCγ2-dependent pathway, leading to generation of autocrats that recruit additional platelets to the site of injury. Signaling downstream of the GPVI/Fc receptor γ chain is similar to signaling initiated by activation of immune receptors on T- and B-lymphocytes. The Fc receptor γ chain contains an immunoreceptor tyrosine activation motif. Binding of collagen to the receptor is thought to cluster the receptors, which initiates the phosphorylation of the immunoreceptor tyrosine activation motif tyrosines by an Src family kinase. Similar to signaling through other immune receptors, Syk tyrosine kinase binds to the phosphorylated immunoreceptor tyrosine activation motif to start a cascade of phosphorylations and protein interactions resulting in the activation of PLCγ2.

Hematopoietic cells express two members of the Cbl family of E3 ligases namely, c-Cbl and Cbl-b (3–5). The Cbl family proteins have been shown to be key regulators of intracellular signaling in immune cells (6, 7). As E3 ligases, Cbl proteins can catalyze the transfer of ubiquitin molecules to their substrates. A primary role for ubiquitylation is targeting the substrates of E3 ligases to the proteasome, where the substrates are proteolytically degraded. Because of this activity, Cbl family members have come to be considered as negative regulators of signaling. In platelets, c-Cbl promotes ubiquitylation of Syk and appears to be a negative regulator of platelet activation. Murine platelets deficient in c-Cbl show enhanced platelet aggregation in response to convulxin, a GPVI agonist (8, 9). Furthermore, we have shown that Syk is not ubiquitylated in c-Cbl−/− platelets, in contrast to normal platelets (9).

The presence of Cbl-b in platelets and any role it might have in signaling have not been investigated. Because Cbl-b is also an E3 ligase, one might expect that it would also be a negative regulator of cell function. However, several reports have indicated that Cbl-b may play a positive role in both T cell and B cell signaling. Cbl-b interacts with Zap-70, leading to activation of T-cell-specific transcription factor NF-AT (10). Cbl-b−/− DT40 B cells display reduced PLCγ2 activation and Ca2+ mobilization upon B-cell receptor stimulation, and the overexpression of Cbl-b results in an enhanced agonist-dependent Ca2+ mobilization (11). These results may be explained by another function of the Cbl molecules, namely that they have several domains in addition to those responsible for the E3 ligase activity, which, acting as a scaffold, can bind to a variety of signaling molecules, bringing them into close proximity (6, 12, 13).

Given the importance of Cbl-b to regulation of signaling in lymphocytes, we have investigated whether Cbl-b regulates platelet activation downstream of the collagen receptor GPVI. We report that Cbl-b is present in human platelets. Murine platelets deficient in Cbl-b show diminished agonist-dependent platelet aggregation and Ca2+ mobilization. Using the FeCl3 model of vascular injury, we have found the Cbl-b−/− mice exhibit prolonged time to vessel occlusion.
**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents were from Sigma unless stated otherwise. Anti-phosphotyrosine (4G10) was from Upstate USA (Charlottesville, VA). Anti-Syk (4D10 and N19), anti-Cbl-b (H121, G1, and 246C5a), anti-PLCγ2 (Q20), Protein A/G PLUS-agaro, and horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phosphospecific Syk (Tyr\(^{509}/\text{Tyr}^{516}\)), anti-phospho-Akt (Ser\(^{473}\)), anti-phospho-ERK (Thr\(^{202}/\text{Tyr}^{204}\)), anti-ERK, and anti-actin were from Cell Signaling Technology (Beverly MA). Anti-p85 phosphatidylinositol (PI) 3-kinase was from Calbiochem. Anti-BTK(E9), anti-c-Cbl, and anti-SLP76 were from BD Transduction Laboratories (Franklin Lakes, NJ). Anti-phosphospecific BTK (Tyr\(^{551}\)) was from Epitomics (Burlingame, CA). Anti-phosphospecific PLCγ2 (Tyr\(^{753}\) and Tyr\(^{759}\)) was prepared as previously described (14). AYPGKF was custom synthesized at Research Genetics (Huntsville, AL). SuperSignal West Pico chemiluminescent substrate was from Pierce. Convulxin was purified according to the method of Polgár et al. (15). Cross-linked collagen-related peptide (CRP) was synthesized at the University of Cambridge (16).

**Isolation of Human Platelets**—Blood was collected from informed healthy volunteers according to a protocol approved by the Institutional Review Board of Temple University into one-sixth volume of acid/citrate/dextrose (85 mM sodium citrate, 111 mM glucose, 71.4 mM citric acid). Platelet-rich plasma was obtained by centrifugation at 180 \(\times g\) for 15 min at ambient temperature and incubated with 1 mM aspirin for 30 min at 37 °C. Platelets were isolated from plasma by centrifugation at 800 \(\times g\) for 10 min at ambient temperature and resuspended in HEPES-buffered Tyrode’s solution (137 mM NaCl, 2.7 mM KCl, 2 mM MgCl₂, 0.42 mM NaH₂PO₄, 5 mM glucose, 10 mM HEPES (pH 7.4), 0.2% bovine serum albumin, and 0.1 units/ml apyrase). The platelet count was adjusted to 2 \(\times 10^{10}/\)ml.

**Platelet Activation and Lysate Preparation**—500-\(\mu\)l aliquots of platelets were equilibrated at 37 °C in the presence of 200 nM GR144053 (Sigma), a fibrinogen receptor antagonist, to prevent aggregation. Platelets were stimulated with the indicated concentrations of agonists. Reactions were stopped by the addition of an equal volume of cold lysis buffer (150 mM NaCl, 25 mM Tris (pH 7.6), 1% Nonidet P-40, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 100 \(\mu\)M phenylmethysulfonyl fluoride, and 10 \(\mu\)g/ml leupeptin). Samples were kept on ice for 10 min to ensure total lysis and centrifuged either for 10 min at 12,000 \(\times g\) at 4 °C or for 30 min at 100,000 \(\times g\) at 4 °C to make certain that the cytoskeletal proteins were removed.

The following antibodies were from Emfret Analytics (Eibelstät, Germany): phcoerythrin-conjugated rat anti-mouse JON/A, phcoerythrin-conjugated anti-mouse CD41 (allb), fluorescein isothiocyanate-conjugated rat anti-mouse GPIV (clone JAIQ), fluorescein isothiocyanate-conjugated rat anti-mouse integrin α2 (CD49b, GPIa), fluorescein isothiocyanate-conjugated rat anti-mouse GPIb α (CD42b). These were used for flow cytometry. Peripheral blood mononuclear cells (PBMCs) were isolated from human blood using Ficoll-Paque Plus (Amersham Biosciences) according to the manufacturer’s instructions.

**Preparation of Mouse Platelets**—Cbl-b\(^{-/-}\) mice were a gift of Drs. Hua Gu and Richard Hodes, and generation of these mice has been reported (17). Generation of Cbl-b RING finger mutant has also been previously documented (18). Mutant mice and WT littermates were 8–10 weeks of age on a C57BL/6 genetic background. All mice were maintained and housed in a specific pathogen-free facility, and animal procedures were carried out in accordance with institutional guidelines after the Temple University Animal Care and Use Committee approved the study protocol. Whole blood platelet counts of these mice were determined using a Hemavet 950FS blood cell counter (Drew Scientific Inc., Dallas, TX). All mice tested were within the normal range.

For aggregation, blood was collected from the vena cava of anesthetized mice into syringes containing 1:10 blood volume of 3.8% sodium citrate as anticoagulant. The blood was then mixed with 200 \(\mu\)l of 3.8% sodium citrate, and red blood cells were separated by centrifugation at 100 \(\times g\) for 10 min. Platelet-rich plasma was removed, an additional 400 \(\mu\)l of 3.8% sodium citrate was added to the residual red blood cells, and cells were centrifuged again at 100 \(\times g\) for 10 min. The platelet-rich plasma was combined, 1 \(\mu\)M prostaglandin E\(_1\) was added, and samples were centrifuged at 400 \(\times g\) for 10 min. The platelet pellet was resuspended in Tyrode’s buffer (pH 7.4) containing 0.1 unit/ml apyrase. Aggregation was performed in a lumiaaggregometer (Chrono-Log Corp., Havertown, PA).

Murine platelets for measurement of calcium mobilization were prepared differently. Whole blood was mixed with 4 volumes of PIPES-buffered Tyrode’s solution (pH 6.5) containing 500 \(\mu\)M EGTA, 10 \(\mu\)M indomethacin, and 1 \(\mu\)M prostaglandin E\(_1\). The mixture was centrifuged for 15 min at 100 \(\times g\) at room temperature. The platelet-rich plasma was removed, diluted to 10 ml with the above buffer, and centrifuged at 800 \(\times g\) for 15 min at room temperature. The platelet pellet was resuspended in the above buffer (1 ml/mouse) and incubated with 5 \(\mu\)M Fura-2/AM for 45 min at room temperature. The platelets were centrifuged at 800 \(\times g\) for 10 min at room temperature and finally resuspended in HEPES-buffered Tyrode’s solution containing 10 \(\mu\)M indomethacin. The platelets were allowed to recover for 15 min at room temperature prior to the start of the experiment. The details of Ca\(^{2+}\) measurement have been previously documented (19). The data in the graphs represent the maximal Ca\(^{2+}\) concentration minus basal Ca\(^{2+}\) concentration.

**Measurement of Platelet Secretion**—Platelet secretion was determined by measuring the release of ATP using the Chro- nolume reagent (Chrono-Log). The activation of platelets was performed in a lumiaaggregometer at 37 °C with stirring at 900 rpm, and the secretion was measured and expressed as pmol of ATP released per 10\(^8\) platelets.

**Flow Cytometry**—Washed murine platelets were used to measure agonist-dependent level of activated αIbb3 receptors by JON/A-phcoerythrin antibody. Aliquots (0.3 ml) of washed platelet suspension in Tyrode’s buffer (pH 7.4) containing 1 mM CaCl\(_2\) were preincubated with the indicated concentrations of convulxin for 5 min at 37 °C. An aliquot containing 10\(^6\) platelets was gently mixed with 10 \(\mu\)l of antibody mixture and incubated...
for 15 min at 37 °C in the dark. Platelets were identified and gated according to the forward and side scatter signal during dual color analysis. As a control for immunolabeling, platelets were incubated with nonimmune IgG isotype control antibody. To fix the platelets, 1% paraformaldehyde dissolved in phosphate-buffered saline was added. A total of 10,000 platelet events were acquired per sample, and the percentage of positive gated cells was analyzed.

For surface expression of cllb (CD41), GPVI (clone JAQ1), α2 (CD49b, GPIa), and GP Ibα (CD42b), aliquots (30 µl) of murine washed platelet suspension in Tyrode’s buffer (pH 7.4) containing 1 mM CaCl2 were incubated with 10 µl of the corresponding antibody for 15 min at 37 °C in the dark and analyzed as described above. All determinations were performed on a FACS Calibur flow cytometer (BD Biosciences) (20).

Immunoblotting—Proteins were subjected to SDS-PAGE (8% acrylamide) and transferred to Immobilon-P. Blots were blocked with 5% nonfat milk in TBS-T (TBS + 0.05% Tween) for 1 h at room temperature and probed overnight at 4 °C with appropriate antibodies. Blots were washed three times with TBS-T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 60 min at room temperature. Blots were washed an additional three times, and antigen-antibody complexes were detected using a chemiluminescent substrate. Bands were visualized on a Fuji imaging system, and densities were calculated with Image Gauge software.

For detection of phospho-Syk, phospho-BTK, and phospho-PLCγ2 in murine platelets, samples were aggregated with varying concentrations of convulxin for 1 min in the presence of indomethacin and the ADP antagonists MRS2179 (100 µM) and 2-methio-AMP (5 µM). An equal volume of 10 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA was added, and the sample was centrifuged for 2 min at 800 × g at room temperature. The supernatant was removed, and the platelet pellet was solubilized in 100 µl of Laemmli reducing sample buffer and boiled for 10 min. Alternatively, proteins were precipitated with 0.6 N perchloric acid, the precipitates were washed once with water, and the protein pellets were solubilized in 100 µl of Laemmli reducing sample buffer and boiled for 10 min. Proteins were separated and detected as described above. For phospho-AKT, phospho-ERK, and total ERK, platelets were treated in the same manner, and blots were analyzed using the Licor Odyssey imaging system.

Human platelets were isolated as described above except that one wash with PIPES-buffered Tyrode’s solution (pH 6.5) containing 500 µM EGTA, 10 µM indomethacin, and 1 µM prostaglandin E1 was included prior to final resuspension. The platelet count was adjusted to 4 × 106/ml, and 2-ml aliquots were activated in the presence of 200 nM GR144053 at 37 °C with 200 ng/ml convulxin for the indicated periods of time. The reaction was stopped by the addition of an equal volume of ice-cold lysis buffer, resulting in final concentrations of 150 mM NaCl, 25 mM Tris (pH 7.6), 1% Nonidet P-40, 0.1% SDS, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM 4-(2-aminoethyl)-benzenesulfonfyl fluoride, and 10 µg/ml leupeptin. Debris was removed by centrifugation at 12,000 × g for 10 min at +4 °C. Supernatants were transferred to clean tubes, and 50 pmol of GST-Cbl-b-agarose or GST-agarose were added. Samples were rocked for 60 min at 4 °C, and the agarose beads were washed three times with lysis buffer and once with Tris-buffered saline. Solubilized proteins were separated by SDS-PAGE and probed for specific proteins as indicated.

For co-immunoprecipitation studies, lysates were prepared in a manner similar to that described above except that centrifugation was for 30 min at 100,000 × g at 4 °C. Proteins were immunoprecipitated for 16 h at 4 °C with 2 µg of appropriate antibodies. 25 µl of Protein A/G PLUS-agarose were added, and samples were incubated for another 60 min at 4 °C with rocking. Immunoprecipitates were washed three times with 1× lysis buffer and one time with TBS (10 mM Tris (pH 7.6) and 150 mM NaCl).

GST-Cbl-b Pull-down Assay and Coimmunoprecipitation—Full-length human Cbl-b cDNA (Thermo Scientific Open Biosystems, Huntsville, AL) was inserted into the polycloning site (Xhol-KpnI) of the baculovirus vector pAcGHLT-B (Pharmingen, San Diego, CA). SF9 cells were co-transfected with the full-length Cbl-b clone and BaculoGold DNA, the recombinant virus was amplified, and the GST-Cbl-b recombinant protein was expressed and purified according to the manufacturer’s instructions.
blood flow during the application of FeCl₃.) Time to thrombotic occlusion after initiation of arterial injury was defined as the time required for blood flow to decline to 0 ml/min. The operator was blinded to mouse genotype while performing all experiments.

**Statistics**—All statistics were calculated using GraphPad Prism®. Dose-response curves were fit to a simple hyperbolic equation, and differences between the fits of WT and mutant cells were determined using comparisons built into that package.

**RESULTS**

Because Cbl-b has not previously been shown to be present in platelets, we assessed its presence using Western blotting. To show that the platelet expression level of Cbl-b is comparable with that in cells in which the biochemical and physiologic importance of Cbl-b is established, we compared Cbl-b levels in PBMCs with those in platelets by comparing Cbl-b immunoreactivity over a range of protein concentrations. As shown in Fig. 1A, Cbl-b is expressed in platelets at levels similar to its expression in PBMCs. We next determined whether Cbl-b, similar to c-Cbl, is tyrosine-phosphorylated in platelets in response to GPVI agonists. Fig. 1B compares phosphorylation of c-Cbl to Cbl-b in immunoprecipitates of each respective protein in platelets stimulated with convulxin (100 ng/ml). Although c-Cbl shows marked phosphorylation, no significant phosphorylation of Cbl-b is seen, indicating that GPVI-dependent activation does not result in Cbl-b phosphorylation.

In order to address the significance of Cbl-b in intracellular signaling in platelets, we undertook a study of the consequences of Cbl-b deficiency for platelet activation. Prior to these studies, we characterized the effect of Cbl-b gene ablation on the expression of other relevant platelet signaling proteins. Fig. 2 shows that for all of the proteins tested, including c-Cbl, Syk, BTK, GPVI, and PLCγ2, their levels in Cbl-b−/− platelets were not altered compared with WT platelets. We also monitored the levels of GPVI, αIIbβ3, GPla1α, and GPIb on the platelet surface using flow cytometry. We did not find a significant difference when platelets from WT mice were compared with Cbl-b−/− platelets (supplemental Fig. S1).

Although we had shown that c-Cbl is required for the ubiquitylation of Syk, it is possible that Cbl-b might also play a role in Syk ubiquitylation. In order to address this question, we obtained platelets isolated from Cbl-b knock-out mice and then stimulated with the GPVI agonist convulxin. Fig. 3 shows that ubiquitylation of Syk is normal in the Cbl-b−/− murine platelets, as indicated by the presence of the immunoreactive bands at about 8 and 16 kDa above Syk. The same experiment was performed in c-Cbl−/− platelets for contrast because these cells show an absence of the ubiquitylated bands (9).

In addition to being E3 ligases, Cbl family members also have been shown to function as adapter proteins in many different cells. Therefore, we investigated whether Cbl-b might play a similar role in platelets. Previous studies in DT40 cells had
shown that Cbl-b associates with both BTK and PLCγ2 (11). We tried three approaches to test the specificity of interaction of PLCγ2 and BTK with Cbl-b. First, we used co-immunoprecipitation using anti-Cbl-b antibodies. In order to minimize nonspecific interactions, platelet lysates were precleared at 100,000 × g prior to immunoprecipitation to remove cytoskeletal proteins. Samples were treated with either anti-Cbl-b or control IgG in the presence of 0.1% SDS. The blots of immunoprecipitated protein were probed with antibodies to either Cbl-b, BTK, PLCγ2, or Syk. All four proteins were found in these immunoprecipitates (Fig. 4A). However, because Syk was also found in the non-immune control, its interaction with Cbl-b was judged to be nonspecific. Following the same protocol, anti-BTK was used to immunoprecipitate proteins from platelet lysates. Again, both Cbl-b and PLCγ2 were found to specifically associate with BTK (Fig. 4B). Last, a GST pull-down experiment was performed using GST-Cbl-b. Precleared lysates were treated with either GST-agarose or Cbl-b-GST-agarose in the presence of 0.1% SDS. Fig. 4C shows that PLCγ2 and BTK from human platelets specifically associate with Cbl-b. The specificity of the reaction is indicated by the fact that GST-agarose does not associate with either protein. In contrast, Syk does not appear to be specifically associated with Cbl-b, confirming the co-immunoprecipitation experiments. Overall, the experiments suggest that the interactions of Cbl-b with PLCγ2 and BTK are constitutive because their association is not dependent on agonist stimulation. We also were unable to detect a specific association of Cbl-b with SLP-76 (data not shown).

In order to determine whether Cbl-b plays a physiologically significant role in platelet function, we compared platelet aggregation in WT and Cbl-b−− murine platelets. Fig. 5A shows the comparison of murine platelet aggregation induced
by either convulxin or CRP. With low concentrations of both GPVI agonists, a diminished aggregation is apparent in the Cbl-b−/− platelets. Aggregation in mutant (Cblb-RFm) platelets in which the ring finger domain is altered to inhibit E3 ligase activity (Cbl-b:C373A) (18) did not significantly differ from WT platelets. We also assessed integrin aIIa/IIIb activation using flow cytometry with the JON/A antibody, which detects the active state of this integrin (21). The pattern of integrin activation was similar to that of aggregation with JON/A binding in the Cbl−/− platelets (supplemental Fig. S2).

Secretion of ATP was also assessed in the three different mouse models. A significant reduction was seen in the Cbl−/− platelets but not the Cbl-b-RFm platelets (Fig. 5B). We also assessed GPVI-dependent Ca2+ mobilization in these platelets. Fig. 5C shows representative CRP concentration response Ca2+ traces from a single experiment. We found no difference in either aggregation or secretion induced by the PAR4 agonist, AYPGKF (not shown), Fig. 5, D and E, shows composite concentration-response curves of Ca2+ mobilization for convulxin and CRP from multiple experiments. Both curves show inhibition of Ca2+ mobilization in platelets deficient in Cbl-b. Statistical analysis indicates that the WT and Cbl-b−/− curves were significantly different from each other ($p < 0.0001$ and $p = 0.0033$; CRP and convulxin, respectively). We also determined Ca2+ mobilization induced by the PAR 4 agonist AYPGKF in WT and Cbl-b−/− mice (Fig. 5F). Comparison of responses of WT and Cbl-b−/− platelets for this agonist did not show statistical significance ($p = 0.453$). As a further control (Fig. 5G), we compared Ca2+ mobilization in Cbl-b RFm platelets. Ca2+ mobilization in these platelets is not significantly different from that in WT platelets ($p = 0.101$).

In order to discern the signaling pathways involved in Cbl-b-dependent activation, we compared the activation of key signaling molecules in the GPVI-dependent activation pathway. In Fig. 6A, we have used a phosphospecific antibody to the phosphotyrosines in the activation loop of Syk (Tyr509/Tyr510) and found that there was no difference between WT and Cbl-b−/− cells when stimulated with convulxin ($p = 0.564$). When the activation-dependent phosphorylation of BTK (Tyr551) was determined in the same experiment, Cbl-b−/− mice showed a distinct suppression of BTK phosphorylation (Fig. 6B) that was statistically significant ($p = 0.0001$). Similar results were seen for phospholipase Cγ2 activation (Tyr735/Tyr739; Fig. 6C, pY753,759) ($p = 0.0007$), indicating that Cbl-b facilitates the activation of BTK and PLCγ2 but not Syk. In order to determine whether the reduced PLCγ2 activation of Cbl-b−/− platelets could be explained by a Cbl-b-dependent activation of PI-3 kinase, we compared Akt and ERK activation in WT and Cbl-b−/− cells as shown in Fig. 6D. Because Akt activation is downstream of PI-3 kinase activity, these data indicate that Cbl-b regulates PLCγ2 activation independently of PI-3 kinase activation.

In order to assess the role of Cbl-b in the in vivo function of platelets, Cbl-b−/− mice were studied in the FeCl3 injury model of thrombosis. In this model, the time to occlusion of the carotid artery is assessed by measuring the time until blood flow stops. This assay was chosen because of its greater dependence on exposed collagen. Fig. 7A shows the results of experiments on seven WT mice and 14 Cbl-b−/− mice. The WT mice had a time to occlusion of $6.1 \pm 2.2$ min. This result is relatively standard for WT mice in this assay (22, 23). The Cbl-b−/− mice, in contrast, showed a wide range of time to occlusion. Four mice failed to form an occluding thrombus within the 30-min time limit of the assay, whereas others were similar to WT. Overall, the average time for Cbl-b−/− mice was 16.2 ± 10 min. The increase in time to occlusion in the Cbl-b−/− mice was statistically significant as assessed using Student’s unpaired t test with a greater than 99.5% confidence. Several of the mice that formed a thrombus at an intermediate time (about 15 min) showed resumption in flow after a time that is most likely due to embolization. Fig. 7B compares the thrombus stability in these experiments. The WT mice produced thrombi that were much less likely to embolize. We also measured tail bleeding times but were not able to find a statistically significant difference in the assay (data not shown). The disparity between tail bleeding assay and FeCl3 injury assay has been reported previously in many publications (24–27).

DISCUSSION

The Cbl family proteins, c-Cbl and Cbl-b, have been shown to regulate signaling in a wide variety of cells (6, 7). c-Cbl is phosphorylated downstream of antigen receptors in lymphocytes (28, 29). Multiple protein-tyrosine kinases have been shown to phosphorylate c-Cbl (30–32), although the molecular basis of this phosphorylation is not entirely clear. The specificity of Cbl-b phosphorylation is less defined. Cbl-b has been shown to be tyrosine-phosphorylated upon the engagement of the T-cell receptor (33) and B-cell receptor activation (11). Yasuda et al. (11) have shown that both Syk and Lyn are required for phosphorylation of Cbl-b in B-cells, and because Syk is not required for the tyrosine phosphorylation of c-Cbl in B-cells (34), the authors concluded that phosphorylation of Cbl-b and c-Cbl are regulated differentially.

Although the presence of c-Cbl in platelets is well established, we have shown for the first time that Cbl-b is also expressed in platelets. Because c-Cbl has been shown to be phosphorylated when platelets are stimulated with thrombopoietin (35), convulxin, CRP, and collagen (9, 15), we decided to determine whether Cbl-b is also phosphorylated by GPVI-dependent agonists. Cbl-b has two phosphorylation sites in the C-terminal region that are partially homologous to those of c-Cbl (5). The fact that Cbl-b is not phosphorylated upon platelet activation is at first surprising. However, although robust c-Cbl phosphorylation, apparently catalyzed by either Src fam-

FIGURE 5. Effect of Cbl-b deficiency on platelet responses. A, platelets were prepared from WT, Cbl-b−/−, or Cbl-b-RFm mice for aggregation and stimulated with the indicated concentrations of convulxin or CRP. B, platelet ATP secretion was measured in WT, Cbl-b−/−, or Cbl-b RFm platelets. A and B, representative traces from at least three experiments. C, representative traces from at least three experiments. D–F, agonist-dependent Ca2+ mobilization experiments. D–F, agonist-dependent Ca2+ mobilization from WT or Cbl-b−/− platelets in response to various doses of CRP ($n = 4$) (D), convulxin ($n = 6$) (E), or AYPGKF ($n = 6$) (E). G, platelets were prepared from WT or Cbl-b RFm mice, and CRP-induced Ca2+ mobilization ($n = 3$) was measured. Bars (A–C), 1 min. Error bars (D–G), S.E.
ily kinases or Syk, occurs downstream in most tyrosine kinase-dependent signaling cascades. Bustelo et al. (36) showed that fibroblasts expressing epidermal growth factor or platelet-derived growth factor receptors failed to phosphorylate Cbl-b. Thien and Langdon (7) obtained the same result in the case of T-cell receptor activation of thymocytes and pointed out several other systems in which Cbl-b is not phosphorylated after cellular activation. Because Cbl-b appears to be phosphorylated...
by the same kinases as c-Cbl (7), the reason for this difference is unclear.

The physiological role of Cbl family members in cell functions seems to be cell type-specific. Negative regulation of cellular functions occurs through ubiquitylation of proteins with the concomitant proteolysis (6, 7, 13, 37). In platelets, c-Cbl is required to ubiquitylate Syk, but this ubiquitylation does not lead to proteolysis (9). However, c-Cbl clearly acts as a negative regulator of platelet function, because c-Cbl−/− platelets have enhanced responses to GPVI-dependent activation. Preliminary evidence suggests that c-Cbl can enhance the dephosphorylation of Syk. Cbl-b has no apparent role in the ubiquitylation of Syk (Fig. 3).

In order to define the molecules involved in Cbl-b-dependent activation, we determined whether key signaling proteins co-associate with Cbl-b. Previous studies in the B-lymphocyte cell line, DT40, showed that Cbl-b associates with PLCγ2 and BTK. We have found a similar pattern of association in platelets (Fig. 4). Yasuda et al. (11) have interpreted their data to indicate that Cbl-b acts as a scaffolding molecule bringing PLCγ2, BTK, and possibly other signaling molecules into required proximity. This scaffolding function of Cbl family members is well documented in the literature, and it is probably regulated through the Src homology 3 domain of both PLCγ2 and BTK with the proline-rich region of Cbl-b (6, 12, 13). In this study, we have focused specifically on the interaction of BTK and PLCγ2 with Cbl-b based on mechanistic considerations and the studies of Yasuda et al. (11). However, we did look for an interaction Cbl-b with SLP76 and failed to find a specific interaction (data not shown).

Physiological experiments with Cbl-b−/− platelets (Fig. 5) indicate that Cbl-b is a positive regulator of platelet activation. The dose response of platelet aggregation to GPVI agonists is shifted to the right, but maximal aggregation can occur if the concentration of agonist is sufficient. Although this indicates that Cbl-b-dependent pathways can initiate platelet aggregation, it is likely that parallel pathways exist that take over in the absence of Cbl-b. Supporting this conclusion is the finding that

cating that Cbl-b functions primarily downstream of collagen-dependent platelet activation. This concept is consistent with the fact that this pathway leads to activation of PLCγ2, whereas the G-protein-coupled thrombin receptor causes activation of PLCβ.

In order to support these findings, we investigated the activation of signaling molecules downstream of GPVI activation using phosphospecific antibodies to assess the activation state of each protein. Syk activation did not differ significantly between WT and Cbl-b−/− platelets. This result is consistent with findings that Syk activity and phosphorylation were not different in WT versus Cbl-b−/− DT40 cells (11). There was no difference found in tyrosine phosphorylation of the Syk-related Zap-70 in activated T cells from Cbl-b−/− mice (17, 42). In contrast, we find that activation of both BTK and PLCγ2 is impaired in Cbl-b−/− platelets (Fig. 5, C–E).

To examine the physiological relevance of these in vitro effects on platelet function, we measured the effect of a deficiency of Cbl-b on formation of a platelet thrombus using the ferric chloride model of vascular injury. We chose this model because it causes denudation of the endothelial cell layer and allows interaction with the subendothelial matrix with platelets. Therefore, this treatment is considered a reasonable model of thrombosis (43, 44). Although other models often damage endothelial cells but do not cause them to detach from the vessel wall to expose collagen, recent studies, published after these studies were completed, from Hechler et al. (45) have demonstrated that it is possible to denude the endothelium by laser injury if properly applied. In WT mice, we found that the time to occlusion time is 6.1 ± 2.2 min, which is reasonably consistent with other data (46). In Cbl-b−/− mice, the time to occlusion was much more variable, and the reasons for this are not totally clear. However, there was a statistically significant increase in time to occlusion to 16.2 ± 10 min. In several cases, arteries did not occlude at all within the 30-min time frame of the experiment. In other cases, occlusion occurred, but the thrombus embolized.
Cbl-b in Platelets

Cbl family members negatively regulate several tyrosine kinase-signaling pathways, including collagen-dependent platelet activation (8, 9, 47–52). On the other hand, these proteins can positively regulate many tyrosine kinase-dependent pathways, most likely through the adapter function associated with the C-terminal region of the Cbl molecule. It is in the C-terminal region that c-Cbl and Cbl-b show the greatest divergence of sequence, which can account for different functional between the two proteins. The fact that c-Cbl and Cbl-b regulate different and often opposing function is not unprecedented, and that has been delineated in the case of osteoclasts by us (53, 54). We propose that Cbl-b positively regulates GPVI-dependent activation by functioning as an adapter molecule to bring signaling molecules such as BTK and PLCγ2 together. This interaction would be downstream of Syk activation, consistent with our data indicating that Syk activation is unchanged in Cbl-b null platelets. Recently Braiman et al. (55) proposed that activation of the T-cell antigen receptor leads to the formation of a complex, which is necessary for full activation of PLCγ1. This complex includes LAT, Vav1, SLP76, ITK (a BTK homolog), and c-Cbl. A similar complex has been proposed downstream of GPVI receptor activation, but the possible presence of a Cbl family member has not been recognized (2). Our data indicate that Cbl-b is present in a platelet complex that is required for activation of PLCγ2. In both LAT+/− and Cbl-b−/− murine platelets, stimulation of these cells is less efficient and requires higher GPVI agonist concentration (56), indicating the existence of at least two parallel pathways to activate PLCγ2. The inclusion of several proteins up-regulating the activity of PLCγ2 in the PLCγ2 activation complex may allow signaling to occur albeit less efficiently in the absence of one of these complex members.

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