The Proto-oncogene Product p120<sup>cbl</sup> Links c-Src and Phosphatidylinositol 3’-Kinase to the Integrin Signaling Pathway*

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Integrin-mediated cell adhesion triggers intracellular signaling cascades, including tyrosine phosphorylation of intracellular proteins. We show in this report that p120<sup>cbl</sup> (Cbl), the 120-kDa c-cbl proto-oncogene product, becomes tyrosine-phosphorylated during integrin-mediated macrophage cell adhesion to extracellular matrix substrata and anti-integrin antibodies. This tyrosine phosphorylation does not occur when cells attach to polylysine, to which cells adhere in a nonspecific fashion. It also does not take place when adhesion-induced reorganization of the cytoskeleton is inhibited with cytochalasin D. In contrast to the rapid and transient tyrosine phosphorylation of Cbl by CSF-1 stimulation, tyrosine phosphorylation of Cbl by cell attachment was gradual and persistent. Tyrosine-phosphorylated Cbl was found to form complexes with the SH2 domain-containing signaling proteins Src and phosphatidylinositol 3-kinase; in vitro kinase assays demonstrated that these kinases were active in the Cbl complexes following integrin ligand binding. Furthermore, Cbl was found to translocate to the plasma membrane in response to cell adhesion to fibronectin. These observations suggest that Cbl serves as a docking protein and may transduce signals to downstream signaling pathways following integrin-mediated cell adhesion in macrophages.

Cell-extracellular matrix (ECM) interactions play an important role in a variety of biological processes, including cell growth, differentiation, and migration. Integrins compose the major class of receptors used by cells to interact with ECM proteins (1, 2). The integrin family currently consists of over 20 distinct α/β heterodimeric transmembrane receptors, with the combination of a particular α and β subunit determining the ligand specificity (3). Upon ligand binding, integrins form clusters on the cell surface; this clustering takes place at cellular sites termed focal adhesions and leads to the assembly of intracellular multiprotein complexes associated with the actin cytoskeleton (4). Focal adhesions are thought to act not only as structural links between the ECM and the actin cytoskeleton, but also as sites of signal transduction from the ECM; engagement of cell-surface integrins is associated with a rapid tyrosine phosphorylation of several focal adhesion proteins. In fibroblasts, these proteins include the focal adhesion kinase (FAK) (for a review, see Ref. 5), paxillin (6), tensin (7), and p130<sup>crk</sup> (8–11). Following tyrosine phosphorylation, these proteins engage in multiple protein-protein interactions by binding to signaling proteins containing SH2 domains, including Src family kinases, Csk, and the adaptor proteins Grb2 and Crk (11–25). Collectively, these observations indicate that ligand binding by integrins regulates the functions of multiple docking proteins that may transmit signals to downstream pathways.

In this report, we have studied the tyrosine phosphorylation events taking place during integrin-mediated cell adhesion in macrophages. In myeloid cells, adherence to ECM components or ligation of integrins with antibodies results in a rapid induction of multiple inflammatory mediator genes, cytokines, and collagenases and in a modulation of the proliferative capacity and the phagocytic activity of these cells (for reviews, see Refs. 26–28). In parallel to these events, monocyte/macrophage cell adhesion to ECM proteins is accompanied by a rapid increase in protein tyrosine phosphorylation (29); the protein-tyrosine kinase Syk (30) and paxillin (31) have recently been identified to be among these proteins. FAK, although important in integrin signaling in many other cell types, including fibroblasts, may not play a crucial role in macrophages, as it is absent or expressed at low levels in these cells (29, 31, 32). Consistent with these reports, we found in this study that integrin ligand binding does not induce tyrosine phosphorylation of FAK in mouse macrophages. A profound increase in tyrosine phosphorylation of proteins with apparent molecular masses of 120–130 kDa, however, accompanied integrin-mediated cell adhesion. We sought to determine whether p120<sup>cbl</sup> (Cbl) might be among the 120–130-kDa tyrosine-phosphorylated proteins. Cbl, which was originally identified as the cellular homolog of the Cas NS-1 murine leukemia retroviral oncogene v-cbl, is a novel signaling molecule primarily expressed in hematopoietic cells. Cbl lacks any obvious catalytic domains, but it possesses multiple potential tyrosine phosphorylation sites and proline-rich motifs, which could mediate concurrent association with SH2 and SH3 domain-containing polypeptides, respectively. Cbl also has a carboxyl-terminal leucine zipper, a motif known to promote homo- and heterodimerization of other proteins (for a review, see Ref. 33). Thus, Cbl is well suited for a potential role in assembling intracellular signaling complexes. More important, Cbl has been shown to be abundant in macrophages (34, 35) and was recently identified as a major tyrosine kinase substrate following CSF-1 stimulation or engagement of Fcγ receptors in these cells (35–37). We report here that Cbl is a...
The predominant phosphorylated component in macrophages upon cell adhesion and forms complexes with SH2 domain-containing signaling molecules, such as Src and PI 3-kinase. Thus, protein-protein interactions mediated by Cbl may connect integrin signaling to downstream signaling pathways in macrophages.

**EXPERIMENTAL PROCEDURES**

Reagents—RPMI 1640 medium was supplied by Mediatech (Hernando, VA), fetal calf serum was from Tissue Culture Biologicals (Tulare, CA), and glutamine-Pen-Strep was from Irvine Scientific (Santa Ana, CA). Bovine plasma fibrinectin was obtained from the Finnish Red Cross. Vitronectin was purified from human plasma as described (38). Anti-mouse major histocompatibility complex class I H-2 mononuclear antibody was from American Type Culture Collection. Polyclonal rabbit anti-α5β1 and anti-αvβ3 antibodies, which recognize the mouse integrins (10), were from Dr. Erikki Ruoslahti (The Burnham Institute, La Jolla, CA). Rat anti-mouse p1 integrin antibody was from Pharmingen (San Diego, CA). Polyclonal rabbit anti-Cbl antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-PI 3-kinase antibody, anti-FAK antibody, anti-phosphotyrosine antibody pY20, and horseradish peroxidase-conjugated pY20 were from Transduction Laboratories (Lexington, KY). Monoclonal anti-paxillin antibody was from Zymed Laboratories (South San Francisco, CA). Monoclonal anti-Src antibody 327 was obtained from Dr. Joan Brugge (Ariad Pharmaceuticals, Cambridge, MA), and polyclonal anti-Crk antibody was from Dr. Michiyuki Matsuda (University of Tokyo, Tokyo). The GST-SH3 domains of Crk, PI 3-kinase, and Src were from Dr. Hisamaru Hirai (University of Tokyo, Tokyo). The GST-SH2 domain of Src was from Dr. Hisamaru Hirai (University of Tokyo, Tokyo). The GST-SH3 domains of Crk, PI 3-kinase, and Src were from Dr. Stephen Taylor (Cornell University, Ithaca, NY). The binding and blotting experiments were carried out as described (11).

**Preparations of Cell Lysates, Immunoprecipitations, and Immunoblotting**—Cells were washed with ice-cold phosphate-buffered saline and lysed in modified RIPA buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 100 mM NaF, 0.5 mM Na3VO4, 1 mM EDTA, and protease and phosphatase inhibitors as described above) for 10 min and then scraped off and homogenized in a glass homogenizer. 0.25 volume of buffer containing 10 mM NaF, pH 7.5, 0.5 mM Na3VO4, and 100 mM NaCl was added to the supernatant after which the nuclei and unbroken cells were pelleted at 500 × g for 5 min. EDTA was added to the supernatant to a final concentration of 5 mM before spinning at 100,000 × g for 45 min. The resulting supernatant constituted the “cytosolic fraction.” The pellet was resuspended in lysis buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors as described above) and centrifuged at 10,000 × g for 15 min. The supernatant from this step was termed the “membrane fraction.”

**RESULTS**

**Macrophage Cell Adhesion Induces Tyrosine Phosphorylation of Cbl**—To investigate tyrosine phosphorylation of proteins in response to cell adhesion, the mouse macrophage cell lines IC-21, P388D1, and RAW 264.7 were either kept in suspension or plated on fibronectin or on anti-integrin antibodies for 20 or 40 min or onto polysine for 40 min. Where indicated, cells cultured as a monolayer were stimulated with CSF-1 (25 nM) for the indicated times prior the lysis. Cells were lysed in Nonidet P-40 Triton X-100 buffer, and immunoprecipitations with anti-phosphotyrosine antibody, anti-Cbl antibody, and a control unrelated antibody were carried out as described above. To investigate tyrosine phosphorylation of proteins in response to cell adhesion, the mouse macrophage cell lines IC-21, P388D1, and RAW 264.7 were either kept in suspension or plated on fibronectin or on anti-integrin antibodies for 20 or 40 min or onto polysine for 40 min. Where indicated, cells cultured as a monolayer were stimulated with CSF-1 (25 nM) for the indicated times prior the lysis. Cells were lysed in Nonidet P-40 Triton X-100 buffer, and immunoprecipitations with anti-phosphotyrosine antibody, anti-Cbl antibody, and a control unrelated antibody were carried out as described above. To investigate tyrosine phosphorylation of proteins in response to cell adhesion, the mouse macrophage cell lines IC-21, P388D1, and RAW 264.7 were either kept in suspension or plated on fibronectin or on anti-integrin antibodies for 20 or 40 min or onto polysine for 40 min. Where indicated, cells cultured as a monolayer were stimulated with CSF-1 (25 nM) for the indicated times prior the lysis. Cells were lysed in Nonidet P-40 Triton X-100 buffer, and immunoprecipitations with anti-phosphotyrosine antibody, anti-Cbl antibody, and a control unrelated antibody were carried out as described above. To investigate tyrosine phosphorylation of proteins in response to cell adhesion, the mouse macrophage cell lines IC-21, P388D1, and RAW 264.7 were either kept in suspension or plated on fibronectin or on anti-integrin antibodies for 20 or 40 min or onto polysine for 40 min. When indicated, cells cultured as a monolayer were stimulated with CSF-1 (25 nM) for the indicated times prior the lysis. Cells were lysed in Nonidet P-40 Triton X-100 buffer, and immunoprecipitations with anti-phosphotyrosine antibody, anti-Cbl antibody, and a control unrelated antibody were carried out as described above. To investigate tyrosine phosphorylation of proteins in response to cell adhesion, the mouse macrophage cell lines IC-21, P388D1, and RAW 264.7 were either kept in suspension or plated on fibronectin or on anti-integrin antibodies for 20 or 40 min or onto polysine for 40 min. When indicated, cells cultured as a monolayer were stimulated with CSF-1 (25 nM) for the indicated times prior the lysis. Cells were lysed in Nonidet P-40 Triton X-100 buffer, and immunoprecipitations with anti-phosphotyrosine antibody, anti-Cbl antibody, and a control unrelated antibody were carried out as described above.
Tyrosine phosphorylation of Cbl in response to cell adhesion to ECM proteins and anti-integrin antibodies. IC-21 cells were either held in suspension (S, lanes 1, 3, and 10) or plated on dishes coated with fibronectin (Fn; lanes 2, 4, and 11), vitronectin (Vn; lane 5), anti-α5β1 antibodies (lane 6), anti-αvβ3 antibodies (lane 7), polylysine (PLL; lane 8), or anti-major histocompatibility complex antibodies (a-MHC; lane 9). Phosphotyrosine (lanes 1 and 2), Cbl (lanes 3–9), and FAK (lanes 10 and 11) immunoprecipitates (IP) were separated by SDS-PAGE and analyzed by anti-phosphotyrosine (α-pTyr) immunoblotting (upper panel). The blots were reprobed with anti-Cbl (lanes 3–9) and anti-FAK (lanes 10 and 11) antibodies to confirm equal loading (lower panel). The molecular masses of marker proteins are indicated in kilodaltons.

An increase in tyrosine phosphorylation of Cbl was seen in cells plated on fibronectin, vitronectin, or anti-α5β1 or anti-αvβ3 antibodies (lanes 4–7). Cell adhesion to polylysine, to which cells can adhere in a non-specific fashion, had no effect on the tyrosine phosphorylation of Cbl (lane 8). Similarly, no increase in tyrosine phosphorylation was observed when cells were allowed to adhere to dishes coated with anti-major histocompatibility complex antibodies (lane 9). The amount of Cbl was essentially the same in all samples, as shown when the same blot was stripped and reprobed with anti-Cbl antibody (Fig. 1, lower panel).

The phosphorylation of FAK, p130Cas, paxillin, and tensin in fibroblasts has been shown to coincide with cell adhesion, with maximal phosphorylation occurring around the time of cell spreading and actin filament reorganization (6, 7, 9, 10). To study the connection of Cbl phosphorylation with actin filament reorganization, IC-21 cells were allowed to adhere to fibronectin-coated dishes, and tyrosine phosphorylation of Cbl was assessed as a function of time (Fig. 2A). At time 0, Cbl contained little phosphotyrosine. Tyrosine phosphorylation of Cbl was detectable after 10 min of adhesion. After 20 min, a clear increase in Cbl phosphorylation was observed in cells plated on fibronectin; at this time point, most of the cells had adhered to fibronectin, but had not yet fully obtained a flat, spread morphology. Maximal tyrosine phosphorylation of Cbl occurred at 40 min after plating when macrophages appeared maximally spread on fibronectin. After 90 min, phosphorylation slowly declined and remained unchanged after 5 h (300 min) of plating under serum-free conditions.

To determine whether reorganization of the cytoskeleton is necessary for cell adherence to stimulate tyrosine phosphorylation of Cbl, the spreading of cells plated onto a fibronectin substrate was inhibited with cytochalasin D; cytochalasin D treatment has been shown to prevent integrin-mediated tyrosine phosphorylation of p130Cas, FAK, and paxillin (7, 9, 10). Cytochalasin D prevented cell spreading and tyrosine phosphorylation of Cbl in a dose-dependent manner. At a cytochalasin D concentration of 0.05 μM, no effect was observed on cell spreading or on tyrosine phosphorylation of Cbl (Fig. 2A, right panels). Cytochalasin D at a concentration of 0.25 μM clearly decreased the integrin-induced tyrosine phosphorylation of Cbl; this decrease correlated with the inhibition of cell spreading. A complete inhibition of both cell spreading and adhesion-induced Cbl phosphorylation was observed when cells were treated with 1.0 μM cytochalasin D.

In contrast to the gradual and persistent tyrosine phosphorylation of Cbl observed above during cell adhesion to fibronectin, CSF-1 stimulation has earlier been shown to result in a rapid and transient tyrosine phosphorylation of Cbl in macrophages (36). Indeed, anti-phosphotyrosine immunoblotting of anti-Cbl immunoprecipitates from cell lysates prepared at various times after CSF-1 stimulation of IC-21 cells showed that Cbl is rapidly and transiently (peak at 1–3 min) tyrosine-phosphorylated. Cytochalasin D treatment did not affect the tyrosine phosphorylation of Cbl following CSF-1 stimulation (Fig. 2B). These results demonstrate that both integrin- and growth factor-mediated signaling pathways stimulate tyrosine phosphorylation of Cbl in macrophages and that these pathways appear to be mechanistically different.

Interaction of Cbl with SH2 Domain-containing Signaling Molecules in an Adhesion-dependent Manner—The tyrosine-phosphorylated sites in Cbl may function as binding sites for proteins containing SH2 domains, as is the case with the other integrin-regulated proteins, p130Cas, FAK, and paxillin (see the Introduction). Tyrosine phosphorylation of Cbl has been de-
FIG. 3. Cbl interacts with SH2 domain-containing signaling molecules, such as Src and PI 3-kinase, in an adhesion-dependent manner. A, RIPA cell lysates were prepared from IC-21 cells adherent to fibronectin (Fn), held in suspension (Susp), or plated on polylysine (PLL). Lysates were incubated with the indicated GST-SH2 domain fusion proteins coupled to glutathione-agarose, and precipitates were analyzed by immunoblotting with anti-Cbl antibody. B, RIPA lysates were prepared from cells adherent to fibronectin or held in suspension (S) as described for A. The lysates were incubated with the indicated GST-SH3 domain fusion proteins, and precipitates were analyzed by anti-Cbl immunoblotting. C, cell lysates from suspended or fibronectin-adherent cells were immunoprecipitated (IP) with antibodies against PI 3-kinase (PI-3-K), Src, or Cbl and analyzed by anti-Cbl immunoblotting. The blot was reprobed with anti-PI 3-kinase and anti-Src antibodies to confirm equal loading (data not shown). D, boiled RIPA lysates were immunoprecipitated with anti-Cbl antibody and blotted with the indicated GST-SH2 domain and GST-SH3 domain fusion proteins followed by anti-GST antibody and horseradish peroxidase-conjugated anti-mouse IgG incubation (upper panels). The blots were stripped and reprobed with anti-Cbl antibodies (lower panels).

scribed in response to receptor occupancy of a limited number of receptors in hematopoietic cells; in these systems, phosphorylated Cbl has been shown to interact with signaling molecules by binding their SH2 domains (see “Discussion”). To test whether SH2-containing proteins would bind to Cbl in an adhesion-dependent manner, binding experiments with various GST-SH2 fusion proteins were conducted. As shown in Fig. 3A, the SH2 domains of several signaling proteins bound to Cbl in lysates of IC-21 cells that had been plated on fibronectin for 40 min. No SH2 domain binding to Cbl was seen in cell lysates prepared from suspended cells or from cells plated on polylysine. Among the signaling molecules found to interact with Cbl in a manner dependent on integrin ligand binding were Src, PI 3-kinase, and Src. No interaction was detected between Cbl and the SH2 domain of Shc.

It has been demonstrated previously that the SH3 domains of a limited number of signaling molecules can bind proline-rich regions of Cbl; these proteins include members of the Src family kinases (37, 45–47) and PI 3-kinase (48, 49). Consistent with these observations, the GST-SH3 domains of Src and PI 3-kinase, but not of Crk, bound to Cbl in IC-21 cell lysates (Fig. 3B). Unlike the SH2 domain interactions described above, the SH3 domain interactions were adhesion-independent since the GST-SH3 domains precipitated Cbl equally well from suspended and adherent cells.

The above results suggest the potential presence of one or more multimeric protein complexes containing Cbl, Crk, Src, and PI 3-kinase; we next carried out coimmunoprecipitation experiments to determine whether Cbl associates with Crk, Src, and/or PI 3-kinase in IC-21 cells. Cell lysates prepared from IC-21 cells either kept in suspension or plated on fibronectin for 40 min were subjected to immunoprecipitation analysis with antibodies against Crk, Src, PI 3-kinase, and Cbl. Immunoprecipitates were analyzed by immunoblotting with anti-Cbl antibodies. As shown in Fig. 3C, Cbl coprecipitated with Src and PI 3-kinase from fibronectin-adherent IC-21 cells. We estimate that under our experimental conditions, 10% of cellular Cbl is associated with Src and PI 3-kinase following integrin-mediated cell adhesion. Little coprecipitation was detected in samples prepared from suspended IC-21 cells. Likewise, Src and PI 3-kinase, but not Crk, were detected in anti-Cbl antibody immunoprecipitates from adherent cells, but not from suspended cells (see below). No coprecipitation between Crk and Cbl was detected under our experimental conditions (data not shown).

Our results demonstrate that Cbl associates with Src and PI 3-kinase and that this association is markedly increased upon integrin-mediated cell adhesion. Our results do not indicate, however, if this interaction and binding of the SH2 or SH3 domains of Src and PI 3-kinase to Cbl are direct or indirect. To determine whether the association between Cbl and Src and between Cbl and PI 3-kinase is direct, Cbl was immunoprecipitated from suspended and fibronectin-adherent (40 min) IC-21 cells and subjected to an overlay assay using GST-Src SH2, GST-Src SH3, GST-PI-3-kinase SH2, and GST-PI-3-kinase SH3 fusion proteins as probes. As shown in Fig. 3D, a 120-kDa protein was detected by all the fusion proteins on the lanes of anti-Cbl precipitates from adherent cells; SH3 domain fusion proteins recognized the 120-kDa protein also from suspended cells. No protein was detected when control precipitates with irrelevant antibodies were probed with the fusion proteins or when anti-Cbl immunoprecipitates were probed with GST-Shc SH2 (data not shown). The 120-kDa protein detected in the anti-Cbl immunoprecipitates by the fusion proteins had the same mobility as Cbl detected by reprobing of the membranes with anti-Cbl antibody (Fig. 3D).

These results indicate that the SH2 and SH3 domains of Src and PI 3-kinase bind directly to Cbl. The SH3 domains of Src and PI 3-kinase bind to Cbl in both suspended and adherent cells; however, coprecipitation of Src and Cbl as well as PI 3-kinase and Cbl was observed only in adherent cells. Therefore, it appears that the SH3 domain binding alone is not sufficient to result in an efficient coprecipitation of these proteins with Cbl. Instead, a direct binding of the SH2 domains of Src and PI 3-kinase to phosphorylated Cbl, which takes place in adherent cells, may be required for the stable complex formation.

Association of Src and PI 3-Kinase Enzymatic Activities with Cbl—The interaction of Src and PI 3-kinase with Cbl in adherent cells suggested a mechanism to recruit Src and PI 3-kinase enzymatic activities into Cbl signaling complexes following integrin ligand binding. To examine if this is the case, we measured the in vitro kinase activity and the PI 3-kinase activity associated with anti-Cbl immunoprecipitates from suspended and adherent IC-21 cells.

Anti-Cbl immunoprecipitates were incubated in kinase assay buffer containing \([γ-32P]ATP\) to reveal any associated kinase activity; Cbl itself lacks any known enzymatic activity (33). The samples were analyzed by SDS-PAGE; following electrophoresis, gels were subjected to alkaline hydrolysis, after which tyrosine-phosphorylated proteins were detected by autoradiography. As shown in Fig. 4A, a low level of tyrosine kinase activity was associated with Cbl immunoprecipitates from sus-
Tyrosine kinase activity in Cbl immunoprecipitates was markedly increased upon integrin-mediated cell adhesion to fibronectin for 40 min (third lane); no increase in tyrosine kinase activity was observed in samples prepared from cells plated on polylysine (first lane). Two predominant phosphorylated bands of 120 and 60 kDa were present following the in vitro kinase assay of Cbl immunoprecipitates from fibronectin-adherent cells. To study whether these may represent Cbl and Src, respectively, bound protein was released from the immunocomplex after the in vitro kinase assay, and Cbl and Src were reprecipitated prior to SDS-PAGE analysis (Fig. 4B). Reprecipitation experiments identified Cbl as one of the phosphorylated 120-kDa proteins and Src as one of the 60-kDa proteins present in the in vitro kinase assay complex. To determine whether Src exhibits kinase activity in the Cbl complex, Cbl immunoprecipitates were subjected to the in vitro kinase assay in the presence of enolase, a classic Src family kinase substrate. These experiments demonstrated 5-fold increased kinase activity toward enolase in Cbl immunoprecipitates from adherent cells compared with immunoprecipitates from suspended cells. Tyrosine kinase activity in Cbl immunoprecipitates was markedly increased upon integrin-mediated cell adhesion to fibronectin for 40 min (third lane); no increase in tyrosine kinase activity was observed in samples prepared from cells plated on polylysine (first lane). Two predominant phosphorylated bands of 120 and 60 kDa were present following the in vitro kinase assay of Cbl immunoprecipitates from fibronectin-adherent cells. To study whether these may represent Cbl and Src, respectively, bound protein was released from the immunocomplex after the in vitro kinase assay, and Cbl and Src were reprecipitated prior to SDS-PAGE analysis (Fig. 4B). Reprecipitation experiments identified Cbl as one of the phosphorylated 120-kDa proteins and Src as one of the 60-kDa proteins present in the in vitro kinase assay complex. To determine whether Src exhibits kinase activity in the Cbl complex, Cbl immunoprecipitates were subjected to the in vitro kinase assay in the presence of enolase, a classic Src family kinase substrate. These experiments demonstrated 5-fold increased kinase activity toward enolase in Cbl immunoprecipitates from adherent cells compared with immunoprecipitates from suspended cells (Fig. 4C). In summary, our results suggest that Cbl associates with tyrosine kinase activity following integrin-mediated cell adhesion. Reprecipitation and tyrosine kinase assay experiments using enolase as a substrate suggest that Src, or a Src family kinase, may be at least partially responsible for the Cbl-associated tyrosine kinase activity in adherent cells.

The in vitro lipid kinase assay was carried out to determine whether PI 3-kinase activity becomes associated with Cbl following integrin ligand binding. As shown in Fig. 5, PI 3-kinase activity was negligible in anti-phosphotyrosine immunoprecipitates in IC-21 cells kept in suspension in the absence of any growth factor stimulation. Replating of cells onto polylysine did not stimulate the PI 3-kinase activation in anti-phosphotyrosine immunoprecipitates. Similarly, no PI 3-kinase activity was associated with Cbl complexes in cells kept in suspension or plated on polylysine. Plating of cells on fibronectin or on
anti-integrin antibodies rapidly stimulated the PI 3-kinase activity in IC-21 cells; anti-phosphotyrosine immunoprecipitates from cells plated on fibronectin and on anti-α5β1 antibodies demonstrated a substantial level of PI 3-kinase activity. Similarly, a clearly detectable fraction of PI 3-kinase activity was associated with Cbl complexes in fibronectin-adherent and anti-integrin antibody-adherent cells. The relative levels of Cbl-associated PI 3-kinase activity correlated with the amount of coimmunoprecipitated PI 3-kinase p85 protein (data not shown). Unrelated control antibody did not immunoprecipitate any PI 3-kinase activity from suspended or adherent IC-21 cells (data not shown). As a positive control, we studied PI 3-kinase activation in response to CSF-1 stimulation. CSF-1 stimulation resulted in association of PI 3-kinase activity with the Cbl immunoprecipitates. Maximal PI 3-kinase activity association with Cbl was detected after a 1-min stimulation of the cells with CSF-1; a low level of PI 3-kinase activity was found to be associated with Cbl complexes after 5–10 min of CSF-1 stimulation. This time course correlated with the time course of tyrosine phosphorylation of Cbl observed in response to CSF-1 stimulation (see above). These data suggest that, similarly to growth factor stimulation, integrin-mediated tyrosine phosphorylation of Cbl and the resulting Cbl-PI 3-kinase interaction may provide an important mechanism to recruit PI 3-kinase activity into integrin signaling complexes.

Cbl Translocates to the Membrane in Response to Cell Adhesion to Fibronectin—To gain further insight into the cellular biological responses of Cbl to integrin stimulation, we analyzed the subcellular localization of Cbl in fractionated IC-21 cells plated either on polylysine or on fibronectin. Cells adherent to polylysine or to fibronectin for 40 min were fractionated as described under “Experimental Procedures.” Antiserum against Cbl was used to immunoadsorb Cbl from both cytosol and membrane fractions, and immunoprecipitates were resolved by SDS-PAGE and probed with antibodies against Cbl (Fig. 6, upper panels) and against phosphotyrosine (lower panels). In both polylysine- and fibronectin-adherent cells, the majority of the cellular pool of Cbl was found in the cytoplasmic fraction. However, a 5-fold increase in the concentration of Cbl in the membrane fraction was observed in cells plated on fibronectin compared with cells plated on polylysine (upper right panel), suggesting that Cbl is recruited to the membrane following integrin-mediated cell adhesion. As expected, little tyrosine phosphorylation on Cbl was detected in both cytosol and membrane fractions prepared from cells plated on polylysine, whereas plating of cells on fibronectin stimulated an increase in tyrosine phosphorylation of Cbl in both the cytosolic and membrane fractions (lower panels). Substantially more Cbl protein was immunoprecipitated from the cytosolic fraction than from the membrane fraction (upper panels), based on arbitrary values obtained by densitometric analysis, the stoichiometry of tyrosine-phosphorylated Cbl was higher in immunoprecipitates from the membrane relative to those from the cytosol. The ratio of phosphotyrosine to Cbl protein immunoprecipitated from the membrane was 2.5-fold higher than the ratio within the cytosol in fibronectin-plated cells. Taken together, our biochemical and cellular biological results suggest that integrin-mediated cell adhesion stimulates tyrosine phosphorylation of Cbl and induces the association of a highly tyrosine-phosphorylated pool of Cbl with macrophage membranes.

DISCUSSION

In macrophages as well as in other cell systems, a limited number of proteins have been shown to undergo tyrosine phosphorylation in response to cell adhesion and spreading on extracellular matrix substrata. We show here that p120^BL (Cbl) is a predominant phosphorylated component in macrophages upon integrin-mediated cell adhesion. Upon cell adhesion, Cbl was found to form complexes with SH2 domain-containing signaling molecules, such as Src and PI 3-kinase, and may thus connect integrins to these downstream signaling pathways.

Cbl tyrosine phosphorylation has been reported to occur in response to a number of stimuli, including activation of the T-cell antigen receptor and ligand-induced stimulation of the granulocyte-macrophage colony-stimulating factor-cytokine receptor, the Fcγ receptor, the epidermal growth factor receptor, the CSF-1 receptor, and the B-cell antigen receptor. In addition, Cbl is heavily tyrosine-phosphorylated in v-abl- and bcr-abl-transformed cells (see Refs. 33, 46, 48, and 50–52). In these systems, tyrosine-phosphorylated Cbl has been shown to interact with the SH2 domains of Src family kinases (45, 46), PI 3-kinase (48, 49, 51, 53–55), Crk (52, 56–58), CRKL (59), and Abi (52). Cbl has also been shown to bind SH3 domain-containing signaling molecules, such as the adaptor proteins Grb2 and Nck (34, 46, 48, 51, 53, 60), members of the Src family kinases (37, 45–47), and PI 3-kinase (48, 49). Therefore, Cbl appears to act as a docking protein with the potential of regulating downstream signaling pathways through protein-protein interactions. Our finding that Cbl is part of the integrin signaling cascade indicates that Cbl is a point of convergence in the actions of a variety of factors known to influence cell morphology, locomotion, growth, and differentiation.

Our observation that Cbl becomes tyrosine-phosphorylated following cell adhesion to ECM substrates, but not to polylysine, is consistent with the tyrosine phosphorylation being mediated by integrins. This is further supported by the observation that cell adhesion to different anti-integrin antibodies also results in elevated tyrosine phosphorylation of Cbl. It is not clear how integrin ligand binding initiates activation of intracellular tyrosine kinases; integrins themselves lack any known enzymatic activity, and no direct in vivo association between integrins and tyrosine kinases has been observed (3). Tyrosine phosphorylation of Cbl requires the presence of intact cytoskeleton since the adhesion-induced tyrosine phosphorylation of Cbl can be prevented by cytochalasin D treatment. A similar situation has been observed in other cells with FAK, tensin, and p130^Cas (7, 9, 10). It is therefore possible that kinases associate with integrins through interactions with cytoskeletal complexes induced by cross-linking of integrins, and intact functional cytoskeleton may be required to bring together the various components of this signaling complex.

The structure of Cbl suggests that it is a signal assembly protein; consistent with earlier findings, we found that Cbl can bind in vitro to SH2 and SH3 domains of various signaling molecules. SH3 domains of Src and PI 3-kinase bound Cbl in an adhesion-independent manner, whereas the interactions be-
between Cbl and the SH2 domains of Crk, Src, and PI 3-kinase required integrin-mediated cell adhesion. Coprecipitation of Cbl with Src and PI 3-kinase was observed only in adherent cells, suggesting that a stable complex formation between these proteins may require the SH2 domain binding to phosphorylated Cbl. No coprecipitation between Cbl and Crk was observed under our experimental conditions.

Our finding that Src coprecipitates with Cbl in a kinase-active form suggests that Src might be responsible for the tyrosine phosphorylation of Cbl during integrin-mediated ligand binding. In our model, the binding of Cbl through the Src SH3 domain would have a role in the initial substrate recognition before tyrosine phosphorylation. The Src SH2 domain would reinforce the binding after tyrosine phosphorylation of Cbl, and the tight SH2 domain-mediated association would cause the effective hyperphosphorylation of Cbl by Src tyrosine kinase during integrin-mediated ligand binding. The molecular events leading to the initial step of the tyrosine phosphorylation of Cbl are currently unknown. The SH2 domain-mediated interaction may enzymatically activate Src by releasing the autoinhibition imposed by the interaction between its SH2 domain and a tyrosine-phosphorylated residue near its C-terminal tail; similar activation of Src kinases has been proposed to take place during FAK-Src interaction upon integrin ligand binding (14, 16). Tyrosine phosphorylation of Cbl in turn should allow the recruitment of SH2 domain-containing signaling molecules such as PI 3-kinase and Crk and their associated proteins. This might enable these proteins to be tyrosine-phosphorylated by Src or to interact with the molecules associated with the other domains of Cbl. Thus, Cbl may serve as a docking protein linking Src to downstream signaling molecules in the integrin signaling pathway.

We used enolase as a substrate in the in vitro tyrosine kinase assays in order to detect Src kinase activity in the Cbl immuno-precipitates. Since enolase can be phosphorylated not only by Src, but also by other members of the Src family, it is possible that some of the other family members may contribute to the tyrosine kinase activity observed in Cbl complexes following integrin ligand binding. These kinases include the Src family members Hck, Fgr, and Lyn, which are known to be expressed in myeloid cells (61). Indeed, recent biochemical evidence has implicated some of these kinases in integrin-mediated signaling in myeloid cells (62, 63). Furthermore, examination of bone marrow-derived neutrophils from hck−/− Fgr−/− mice has revealed a severe defect in neutrophil function elicited by plating cells on ECM protein-coated surfaces or by directly cross-linking cell-surface integrins (64). Similar results have recently been seen with monocyes and macrophages derived from hck−/− Fgr−/− double mutant mice (cited in Ref. 61). Our preliminary results suggest that, in addition to Src, Lyn and Fgr may also become associated with Cbl in an integrin-dependent fashion, but a lack of suitable reagents has prevented us from studying these putative interactions in more detail. Together, these results suggest that Src family kinases are involved in integrin signaling in myeloid cells and that Cbl is a candidate molecule in connecting Src kinases to integrin signaling complexes in these cells.

The findings reported here show an in vivo association between Cbl and PI 3-kinase upon integrin ligand binding. In addition, we have demonstrated that a substantial level of PI 3-kinase activity associates with Cbl in an adhesion-dependent manner. PI 3-kinase is known to be activated when one or both of the SH2 domains in the p85 subunit of this enzyme bind to tyrosine-phosphorylated proteins that contain YXXM motifs (65). We found that the N-terminal domain of p85 PI 3-kinase bound to Cbl in an adhesion-dependent manner; Cbl has two YXXM motifs (33) that can serve as binding sites for the SH2 domains of PI 3-kinase. Based on these observations, we postulate that one functional consequence of Cbl tyrosine phosphorylation upon integrin ligand binding is the catalytic activation of PI 3-kinase. Thus, Cbl may play a prominent role in macrophages in coupling the PI 3-kinase enzyme with the integrin signaling machinery. In other systems, activation of PI 3-kinase has been connected to a number of biological effects, such as the mitogenic effects of certain growth factors, changes in actin rearrangement, and growth factor-mediated membrane ruffling and chemotactic migration (for a review, see Ref. 66). Similarly, Cbl-activated PI 3-kinase may be involved in integrin-mediated chemotaxis and phagocytosis in macrophages.

In summary, the ligand binding of integrins seems to control the tyrosine phosphorylation status of a number of intracellular proteins that can function as docking proteins connecting multiple downstream signaling pathways via SH2 and SH3 domain interactions; Cbl appears to mediate integrin signaling through direct recruitment and activation of Src and PI 3-kinase in macrophages. Further studies are needed to define the cause-effect relationships between the tyrosine-phosphorylated proteins, integrins, and actin cytoskeleton and to reveal intermediate steps between integrins and the kinases.

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REFERENCES

1. Hynes, R. O. (1992) Cell 69, 11–25
2. Ruoslahti, E. (1991) J. Clin. Invest. 87, 1–5
3. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) Annu. Rev. Cell Dev. Biol. 11, 549–599
4. Burrage, K., Fath, K., Kelly, S., Nuckolls, G., and Turn, C. (1988) Annu. Rev. Cell Biol. 4, 487–522
5. Clark, E. A., and Brugge, J. S. (1995) Science 269, 233–239
6. Burrage, K., Turner, C. E., and Romer, L. H. (1992) J. Cell Biol. 119, 893–903
7. Beckhoff, S. M., and Burrage, K. (1993) J. Biol. Chem. 268, 14655–14671
8. Petch, L. A., Beckhoff, S. M., Beuton, A., Parsons, J. T., and Burrage, K. (1995) J. Cell Sci. 106, 1373–1379
9. Nojima, Y., Morino, M., Mimura, T., Hamashiki, K., Furuya, H., Sakai, R., Sato, T., Tachibana, K., Monoto, C., Yazaki, Y., and Hirai, H. (1995) J. Biol. Chem. 270, 15398–15402
10. Vuori, K., and Ruoslahti, E. (1994) J. Biol. Chem. 270, 22259–22262
11. Vuori, K., Hirai, H., Aizawa, S., and Ruoslahti, E. (1996) Mol. Cell. Biol. 16, 2606–2613
12. Cobb, S. B., Schaller, M. D., Leu, T. H., and Parsons, J. T. (1994) Mol. Cell. Biol. 14, 147–155
13. Eide, B. L., Furk, C. W., and Escobar, J. A. (1995) Mol. Cell. Biol. 15, 2819–2827
14. Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. K., and Parsons, J. T. (1994) Mol. Cell. Biol. 14, 1600–1608
15. Xing, Z., Chen, H. C., Nowlen, J. K., Taylor, S. J., Shalloway, D., and Guan, J. L. (1994) Mol. Biol Cell 5, 413–421
16. Calab, M., Polte, T. R., and Hanks, S. K. (1995) Mol. Cell. Biol. 15, 954–963
17. Schlaepfer, D. D., Hanks, S. K., Hunter, T., and van der Geer, P. (1994) Nature 372, 786–791
18. Saha, H., Hata, A., Okada, M., Nakagawa, H., and Hanafusa, H. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 3884–3888
19. Schaller, M. D., and Parsons, J. T. (1995) Mol. Cell. Biol. 15, 2635–2645
20. Birge, R. B., Fajardo, J. E., Reichman, C., Shoelson, S. E., Songyang, Z., Cantley, L. C., and Hanafusa, H. (1993) Mol. Cell. Biol. 13, 4648–4656
21. Birge, R. B., Fajardo, J. E., Mayer, B. J., and Hanafusa, H. (1993) J. Biol. Chem. 267, 10588–10595
22. Kannan, S. B., Reynolds, A. B., Wang, H. C., Vines, R. R., and Parsons, J. T. (1991) EMBO J. 10, 1685–1698
23. Petruzzelli, L., Takami, M., and Herrera, R. (1996) J. Biol. Chem. 271, 7796–7801
24. Nakamoto, T., Sakai, R., Ozawa, K., Yazaki, Y., and Hirai, H. (1996) J. Biol. Chem. 271, 8959–8965
25. Bergman, M., Joukov, V., Virtanen, I., and Alitalo, K. (1995) Mol. Cell. Biol. 15, 111–122
26. Mondal, K., Lofquist, A. K., Watson, J. M., Morris, J. S., Price, L. K., and Haskill, J. S. (1995) Biochem. Soc. Trans. 23, 460–464
27. Green, E. J. (1995) Bioessays 17, 199–117
28. Levesque, J. P., Hatzfeld, A., and Hatzfeld, J. (1991) Immunol. Today 12, 258–262
29. Lin, T. H., Yurochko, A., Kornberg, L., Morris, J., Walker, J. J., Haskill, S., and Juliano, R. L. (1994) J. Cell Biol. 126, 1585–1593
30. Lin, T. H., Rosales, C., Mondal, K., Bolen, J. B., Haskell, S., and Juliano, R. L. (1995) *J. Biol. Chem.* **270**, 16189–16197
31. De Nichilo, M. O., and Yamada, K. M. (1996) *J. Biol. Chem.* **271**, 11016–11022
32. Choi, K., Kennedy, M., and Keller, G. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 5747–5751
33. Langdon, W. Y. (1995) *Aust. N. Z. J. Med.* **25**, 859–864
34. Rivero-Lezcano, O. M., Sameshima, J. H., Marcilla, A., and Robbins, K. C. (1994) *J. Biol. Chem.* **269**, 17363–17366
35. Tanaka, S., Neff, L., Baron, R., and Levy, J. B. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 4135–4139
36. Wang, Y., Yeung, Y.-G., Langdon, W. Y., and Stanley, E. R. (1996) *J. Biol. Chem.* **271**, 17–20
37. Marcilla, A., Rivero-Lezcano, O. M., Agarwal, A., and Robbins, K. C. (1995) *J. Biol. Chem.* **270**, 1347–1351
38. Yatohgo, T., Izumi, M., Kashiwagi, H., and Hayashi, M. (1988) *Cell Struct. Funct.* **13**, 281–292
39. Celada, A., and Maki, R. A. (1992) *J. Immunol.* **148**, 1102–1105
40. Vuori, K., and Ruoslahti, E. (1994) *Science* **266**, 1576–1578
41. Jhun, B. H., Rose, D. W., Seely, B. L., Rameh, L., Cantley, L., Saltiel, A. R., and Olefsky, J. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 644–646
42. Hartley, D., and Corvera, S. (1996) *J. Biol. Chem.* **271**, 21939–21943
43. Reedquist, K. A., Fukazawa, T., Druker, B., Panchamoorthy, G., Shoelson, S. E., and Band, H. (1996) *J. Biol. Chem.* **270**, 19141–19150
44. Andoniou, C. E., Thien, C. B. F., and Langdon, W. Y. (1996) *Oncogene* **12**, 1981–1989
45. Hartley, D., Meisner, H., and Corvera, S. (1995) *J. Biol. Chem.* **270**, 18260–18263
46. Reedquist, K. A., Fukazawa, T., Panchamoorthy, G., Langdon, W. Y., Shoelson, S. E., Druker, B. J., and Band, H. (1996) *J. Biol. Chem.* **271**, 8435–8442
47. Berton, G., Fumagalli, L., and Berton, G. (1994) *Cell Struct. Funct.* **19**, 297–331
48. Fukazawa, T., Reedquist, K. A., Trub, T., Soltoff, S., Panchamoorthy, G., Druker, B., Cantley, L., Shoelson, S. E., and Band, H. (1995) *J. Biol. Chem.* **270**, 19141–19150
49. Soltoff, S. P., and Cantley, L. C. (1996) *J. Biol. Chem.* **271**, 563–567
50. Fukazawa, T., Miyuke, S., Band, V., and Band, H. (1996) *J. Biol. Chem.* **271**, 14554–14559
51. Panchamoorthy, G., Fukazawa, T., Miyake, S., Soltoff, S., Reedquist, K., Druker, B., Shoelson, S., Cantley, L., and Band, H. (1996) *J. Biol. Chem.* **271**, 3187–3194
52. Sattler, M., Salgia, R., Okuda, K., Uemura, N., Durstine, M. A., Pasick, E., Xu, G., Li, J.-L., Prasad, K. V., and Griffin, J. D. (1996) *Oncogene* **12**, 839–846
53. Meisner, H., Corvera, S., Hartley, D., and Olefsky, J. M. (1995) *J. Biol. Chem.* **270**, 9115–9120
54. Ribon, V., Hubbell, S., Herrera, R., and Saltiel, A. R. (1996) *J. Biol. Chem.* **271**, 45–52
55. Andoniou, C. E., Thien, C. B. F., and Langdon, W. Y. (1996) *Oncogene* **12**, 839–846
56. Lowell, C. A., Fumagalli, L., and Berton, G. (1996) *J. Biol. Chem.* **271**, 10800–10805
57. Lowell, C. A., and Soriano, P. (1996) *Genes Dev.* **10**, 1845–1857
58. Berton, G., Fumagalli, L., and Berton, G. (1994) *J. Cell Biol.* **126**, 1111–1121
59. Yan, S. R., Fumagalli, L., and Berton, G. (1995) *J. Inflammation* **45**, 297–331
60. Lowell, C. A., Fumagalli, L., and Berton, G. (1996) *J. Cell Biol.* **133**, 895–910
61. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawan, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajarido, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1996) *Cell* **72**, 767–778
62. Kapeller, R., and Cantley, L. C. (1984) *Bioessays* **16**, 556–556