The CBL-related Protein CBLB Participates in FLT3 and Interleukin-7 Receptor Signal Transduction in Pro-B Cells*

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The FLT3 receptor tyrosine kinase and its ligand, FL, play an important role in early hematopoietic development. We have found that CBLB, a recently characterized molecule closely related to the CBL protooncoprotein, is phosphorylated on tyrosine(s) following FL treatment of JEA2 human pro-B cells and THP1 monocytic cells. Treatment of JEA2 cells with interleukin (IL)-7 induces CBLB phosphorylation as well. FL and IL-7, respectively, induce and increase association of tyrosine-phosphorylated SHC and the p85 subunit of phosphatidylinositol 3'-kinase with CBLB. In these cells, CBLB constitutively binds the GRB2 adaptor predominantly through its N-terminal SH3 domain, to form a complex that is distinct from the GRB2-CBL and GRB2-SOS1 complexes. Together with the fact that CBLB is consistently found in blast cells from acute leukemias and in peripheral blood mononuclear cells, this suggests that CBLB has a role in tyrosine kinase-regulated signaling pathways in many hematolymphoid cells.

Hematopoietic differentiation is a complex process that is likely to engage together inductive, enabling, and stochastic events. Phenotypic changes that proceed from stem cells to mature cells of different lineages result from changes in cellular activities such as signal transduction and transcriptional regulation. During these changes, the expression of many molecules is induced or repressed both at the transcriptional and post-transcriptional level. The FLT3 receptor tyrosine kinase (1, 2) and its ligand, FL (3, 4), are potent inducers of monocytye (5) and B cell progenitor (6) production, in combination with cytokines such as colony-stimulating factor 1 and IL-7, respectively. FL mostly regulates cell proliferation and survival (7, 8). In accordance with a role of FLT3 that extends beyond the stem cell level, detectable FLT3 appears first at a low level on the surface of primitive multilineage progenitor cells and disappears during defined stages of B cell development, whereas it is up-regulated and maintained during monocytic differentiation (9). Leukemia inhibitory factor-induced transition from monocytes to macrophages is accompanied by a loss of FLT3 expression in murine M1 cells (10). In maturing murine B cells, FLT3 transcripts are found only in the pre-pro-B and pro-B fractions, and are absent in more mature cells (11). This is in agreement with FL preferentially acting on B220+CD43+CD24- pre-pro-B cells (12).

The FLT3 receptor transduces activation signals through association and/or phosphorylation of cytoplasmic proteins, including RAS GTPase-activating protein, phospholipase C-γ, VAV, and SHC (13, 14). The p85 subunit of phosphatidylinositol 3'-kinase and the RAS pathway-associated GRB2 adaptor associate with the activated receptor through their SH2 domains (14–16). In contrast to the two related receptors, FMS/colony-stimulating factor 1 receptor and KIT, p85PI3K associates with mouse FLT3 at a specific docking site that is located in the C-terminal end of the molecule (14, 15). Recently, we demonstrated that the two prominent FLT3 substrates in human hematopoietic cell lines are p52SHC and p115CRK (17).

The CBL protooncoprotein product is related to the transforming protein of the CAS-NS1 retrovirus that causes pre-B cell lymphomas and myeloid leukemias in mice (18). In the past 4 years, CBL has been proven to be tyrosine-phosphorylated in response to a wide variety of cytokines and growth factors and after engagement of antigen receptors on B and T cells (19–23). Depending on the stimulus and the cell type, CBL binds a number of cytoplasmic enzymatic or regulatory molecules, such as the SYK (24) and ZAP-70 (25) tyrosine kinases and the p85PI3K (22, 26, 27) and CRK adaptors (28, 29).

Recently, a gene product highly related to CBL, named CBLB (30), was characterized. Interestingly, all structural domains found in CBL are also present in CBLB, including an evolutionary conserved N-terminal region, a RING finger-like motif, a proline rich region, and a putative leucine zipper at the C terminus. The former region is able to bind the epidermal growth factor receptor and ZAP-70 in a phosphotyrosine-dependent manner, and could thus define a new structural unit functionally related to the SH2 and phosphotyrosine-binding domains. CBLB may exist as a full-length protein and as shorter isoforms truncated in their C terminus, thus leading to leucine zipper minus proteins (30).

In this paper, we demonstrate that CBLB is expressed in human hematopoietic cell lines of myeloid and B-lymphoid origins that express endogenous FLT3, and in most acute leukemia and peripheral blood cells. In JEA2 pro-B cells, FL or IL-7 exposure induces CBLB phosphorylation on tyrosine residues, its association with a number of tyrosine-phosphorylated molecules including SHC, and increases binding to p85PI3K. Like CBL, CBLB forms a constitutive and SOS-independent complex with the GRB2 adaptor through SH3-dependent interactions. This is the first demonstration that CBLB is a substrate for cytokine-activated tyrosine kinase(s).
EXPERIMENTAL PROCEDURES

Cell Cultures—LAZ221 cells were obtained from the German Collection of Micro-organisms and Cell Cultures, and THP1 and MCF7 cells were obtained from the American Type Culture Collection. JEA2 and OCIAML5 were kind gifts from C. Schiff (Marseille, France) and M. Minden (Toronto, Canada), respectively. All cells were grown in RPMI 1640 supplemented with 10% heat-inactivated (30 min, 56 °C) fetal bovine serum. The culture medium of the growth-factor-dependent OCIAML5 was further supplemented with 10% culture supernatant from the bladder carcinoma cell line 5637.

Leukemic and Peripheral Blood Cells—Pure (>95%) population of leukemic blast cells were prepared from the bone marrow or peripheral blood of leukemic patients with high blast counts (>50 × 10^6/liter) by centrifugation on a Ficoll gradient. All samples were obtained from adult patients with informed consent. Leukemic samples were classified according to French-American-British classification (31) and confirmed by phenotypical analysis.

Peripheral blood cells were isolated from blood samples obtained from healthy donors (Centre Régional de Transfusion Sanguine, Marseille, France) and fractionated into lymphocytes and monocytes as described previously (32).

Antibodies—Anti-phosphotyrosine monoclonal antibody (4G10) and rabbit polyclonal anti-rat p85PI3K were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit polyclonal anti-human CBL (C-15) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GRB2, anti-SOS1, and anti-SHC monoclonal antibody were purchased from Transduction Laboratories (Lexington, KY). A polyclonal anti-human CBLB antibody was obtained by immunization of rabbits with the recombinant C-terminal amino acids of human CBLB (30), coupled to keyhole limpet hemocyanin. For immunoprecipitation experiments, an immunopurified goat polyclonal antibody (C-20, Santa Cruz) made available during the course of our study and directed against the same peptide was used.

Immunodepletions—Five μg of anti-CBL or control rabbit immunoglobulins and 4 μl of anti-CBL or nonimmune rabbit serum were added to 40 μl of the presence of the immunizing peptide diluted in Tris-buffered saline, 0.05% Tween 20. Anti-CBL antibody was shown to express a 4–5-kilobase band in total cellular lysates of two myeloid (THP1 and OCIAML5) and two early-B lymphoid (LAZ221 and JEA2) cell lines known to express FLT3 (36), in addition to MCF7 (Fig. 1A). The same membrane was subsequently immunoblotted with an anti-CBL antibody. The presence of a 115-kDa reactive band in all lanes indicated that CBL and CBLB were coexpressed in all cell lines analyzed. A CBLB doublet was also detected in erythroleukemic HEL and promyelocytic HL60 cell lines (data not shown) and in all acute lymphoid leukemia and AML samples tested, in addition to T and B lymphocytes and monocytes from peripheral blood (Fig. 1B).

RESULTS

CBL and CBLB Proteins Are Coexpressed in Human Cell Lines—To determine whether CBLB could be a substrate for FLT3 or an FLT3-activated tyrosine kinase, we analyzed human hematopoietic cell lines expressing endogenous FLT3 receptors for the presence or absence of product(s) of the CBLB gene. As a first step, we produced an antiserum raised again the C-terminal amino acids of human CBLB that correspond to the C-terminal amino acids of human CBLB (30), coupled to keyhole limpet hemocyanin. For immunoprecipitation experiments, an immunopurified goat polyclonal antibody (C-20, Santa Cruz) made available during the course of our study and directed against the same peptide was used.

Immunoprecipitations—Cloned cell lysates were incubated for 2 h at 4 °C. Immunoprecipitates were pelleted by centrifugation and discarded. Proteins in the supernatants were then recovered by adding 700 μl of cold acetone, followed by centrifugation at 13,000 rpm for 30 min. Protein-containing pellets were resuspended in 25 μl of lysis buffer plus 25 μl of SDS/mercaptoethanol-containing buffer and separated by SDS-PAGE.

Cell Stimulation and Lysis—For Western blot analysis of total cell lysates, cells were starved for 24 h in RPMI 1640 supplemented with 1% heat-inactivated fetal calf serum. Cells were then stimulated or not with 100 μl of Escherichia coli-produced murine FL (3) (kindly provided by DNAX, Palo Alto, CA). Cells were then washed twice in ice-cold phosphate-buffered saline and lysed in 40 μl of 20 μm Tris-HCl, pH 8, 2 mM EDTA, pH 8, 1% Nonidet P-40, 1 mM sodium orthovanadate, 6 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride. Lysates were cleared by centrifugation, heated in SDS/mercaptoethanol-containing buffer, and separated by electrophoresis on polyacrylamide gels.

For immunoprecipitation experiments, cells were starved, stimulated, and washed as mentioned above. Cells were then lysed in 500 μl of lysis buffer.

RIPA lysis buffer is 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate supplemented with protease and phosphatase inhibitors as mentioned above.

Immunoprecipitations—Cloned cell lysates were incubated for 2–4 h at 4 °C with 5 μg of anti-CBL goat antibody (C-20, Santa Cruz) and protein A-coupled Sepharose (Amersham Pharmacia Biotech). Immune complexes were washed 4 times with lysis buffer and then heated in SDS/mercaptoethanol-containing loading buffer and separated by SDS-PAGE.

Western Immunoblotting—Polyacrylamide gels were transferred onto Immobilon membranes (Millipore, St-Quentin-Yvelines, France), and the membranes were probed following the recommendations of the manufacturer with the indicated antibody at a concentration of 1 μg/ml in phosphate-buffered saline, 0.1% Tween 20 except for the 4G10 antibody that was diluted in Tris-buffered saline, 0.05% Tween 20. Anti-CBL rabbit antiserum was used at a 1:500 dilution. Horseradish peroxidase-coupled anti-mouse or anti-rabbit immunoglobulin antibodies (Dako S.A., Trappes, France) were used for detection. For successive probing of the same membrane with different antibodies, stripping was performed in 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.8, for 30 min at 50 °C.

In peptide inhibition experiments, a CBLB-derived or an irrelevant peptide was added to the Western blotting solution at a concentration of 50 μM.

In Vitro Mixing Experiments with GST Fusion Proteins—The induction of bacterially expressed GST fusion proteins was performed as described previously (33). GST fusion proteins were purified from bacterial lysates using glutathione-Sepharose beads (Amersham Pharmacia Biotech). Lysates from nonstimulated or FL-stimulated cells were mixed with 10 μg of recombinant protein on glutathione beads and incubated for 1 h at 4 °C. Protein complexes were washed three times with lysis buffer, resolved by SDS-PAGE, transferred onto Immobilon, and immunoblotted with the appropriate antibodies. GST fusion proteins encoding full-length GRB2 (GST-GRB2) and double SH3 mutant GST-GRB2 49L/203R (named GST-GRB2 μSH3), and isolated amino acid GST-GRB2 SH3 domain (residues 1–58) (N-SH3), and the C-terminal GST-GRB2 domain (residues 159–217) (C-SH3), have been described previously (34, 35) and are kind gifts of D. Cantrell (London, United Kingdom).

CBLB in Pro-B Cells

In pro-B cell lines, CBLB was shown to be phosphorylated at a low to undetectable level in nonstimulated cells, but was phosphorylated on tyrosine residues after 5 min of FL stimulation. We subsequently used only JEA2 cells because they show more CBLB expression and FL-induced tyrosine phosphorylation than THP1 cells.

FL-induced CBLB Phosphorylation Is Transient and Corre-
Convergence of FL and IL-7 Signaling Pathways to CBLB—Because FL plus IL-7 is the most efficient cytokine combination to promote pro-B cells development in vitro, we next analyzed the effect of IL-7 alone or combined with FL on CBLB phosphorylation and associated proteins in JEA2 cells. Fig. 5 shows that IL-7 induced CBLB phosphorylation and association with SHC and p85PI3K in a manner very similar to FL, albeit less intense. No particular change was observed when FL and IL-7 were added together, as compared with FL used alone.

CBLB Binding to GRB2 Is Mediated through the N-terminal GRB2 SH3 Domain—To determine the contribution of individual SH2 and SH3 domains of GRB2 to the association with CBLB, we did in vitro mixing experiments using bacterially expressed GST fusion proteins. Full-length GRB2 was able to bind p115 and p120 CBL products in nonstimulated and FL-activated JEA2 cells (Fig. 6A). Among individual SH domains, only the N-terminal SH3 domain of GRB2 bound CBLB (Fig. 6A). Binding to CBL showed the same domain requirement, as evidenced by subsequent blotting of the same membrane with an anti-CBL antibody.

Distinct GRB2 Pools Selectively Associate with CBL, CBLB, and SOS1, Respectively—Association of GRB2 with SOS1, CBL, and CBLB primarily depends on its N-terminal SH3 domain (see Refs. 23 and 37, and preceding paragraph) and CBL-GRB2 and SOS1-GRB2 complexes are distinct (26, 38). To extend this observation to CBLB-containing complexes, we did immunoblot analysis of anti-SOS1, anti-CBL, and anti-CBLB immunoprecipitates obtained from nonactivated and FL-activated JEA2 cells.

Results shown in Fig. 6B indicate that no cellular SOS1 proteins were present in anti-CBL and anti-CBLB complexes from JEA2 cells. In addition, the anti-CBL immunoblot of the membrane showed no CBL proteins in anti-SOS1 and in anti-CBLB immunoprecipitates from JEA2 cells. Considering the comparable GRB2 contents in anti-CBL, anti-CBLB, and anti-SOS1 immunoprecipitates, we concluded that most if not all CBL, CBLB, and SOS1 molecules were complexed to different pools of GRB2 proteins. FL stimulation did not affect the preformed complexes.

**DISCUSSION**

CBLB is a recently characterized molecule closely related to the product of the CBL protooncogene (30). CBLB and CBL share structural domains including a basic N-terminal region, a RING finger, a proline-rich domain, and a putative leucine zipper. CBL is a cytoplasmic protein that can associate with cellular structures such as the trans-Golgi region and the cytoskeleton (21, 39). In addition, extensive studies have shown that CBL is phosphorylated on tyrosine residues in response to most, if not all, stimuli involving activation of cellular tyrosine kinases, indicating that it could play a central role in controlling signal transduction pathways (40). CBL is constitutively complexed to the GRB2 adaptor through proline-rich/SH3 domain-dependent interactions (19, 23) and inducibly associates with various molecules such as p85PI3K (22, 26, 27) and CRK (26, 28, 29) proteins in an SH2/phosphotyrosine-dependent manner. Despite this, the precise role(s) of CBL are not known, with the recent exception that CBL inhibition blocks SRC-dependent bone resorption (41). We have found that CBL is a major tyrosine-phosphorylated substrate following activation of the FLT3 receptor tyrosine kinase (17). This prompted us to examine whether CBLB could also be involved in FLT3 signaling.

As a first step, we produced CBLB-specific antibodies by immunizing rabbits with a peptide corresponding to the 15 CBLB C-terminal residues. This antiserum was able to detect a protein doublet of 115/120 kDa in cellular lysates of cell lines
known to express FLT3, and in MCF7 breast cancer cells, previously shown to express high quantities of CBLB transcripts (30). Full-length CBLB has a predicted Mr of 104.774. The apparent Mr seen on gel are higher. This difference is likely to result from a high proline content (12.5%) of CBLB, a feature that can maintain secondary structures in denaturing conditions. It is presently not known if the two CBLB polypeptides result from post-translational modification of the same primary product, such as phosphorylation on serine/threonine residues, or if they correspond to isoforms. Two CBLB shorter isoforms, encoded by variant mRNA, lacking 172 and 213 C-terminal amino acids exist (30) but cannot account for the proteins observed on gel, as the immunizing epitope is absent from these two proteins.

Activation of FLT3 by FL leads to tyrosine phosphorylation of CBLB in THP1 monocytic and JEA2 pro-B cells. The relative contribution of the two observed CBLB proteins to the total phosphorylation could not be estimated because of the close proximity of the two bands, relative to the intensity of the tyrosine phosphorylation signal.

The extensive conservation between CBL and CBLB suggests that CBLB, like CBL, is able to form supramolecular complexes with a number of components of the signaling machinery. Indeed, this work shows, for the first time, that it is the case. The association of CBL with GRB2 and p85PI3K are differentially modulated following stimulation of the B and T cell antigen receptors (19–23) and various diffusible factors (20, 42) and adhesion molecules (43). Similarly, CBLB binds GRB2 in a constitutive manner, which is not modified after FL stimulation. The SOS1-derived proline-rich peptide VPPPVPRR binds to GRB2 SH3 domains (37) and is very similar to the sequence SLPPVPPRLD found at position 492 and 503 in CBL and CBLB, respectively. The existence of this site on the two latter proteins is likely to explain their association with GRB2. The binding preference of CBL and CBLB for the N-terminal GRB2 SH3 domain suggests that similar proline-rich sequences, including the one mentioned above, mediate the CBL/CBLB binding to GRB2. 

FIG. 2. Anti-CBL and anti-CBLB antibodies do not show cross-reactivity. A, lysates from 5 × 10^6 JEA2 cells were left untreated (lane 1) or subjected to immunodepletion with anti-CBL (lane 2) or anti-CBLB (lane 3) antibodies (see “Experimental Procedures”). Samples were run on SDS-PAGE, transferred to Immobilon membrane, and immunoblotted with anti-CBL antibody (lower panel). After stripping, the membrane was immunoblotted with anti-CBL antibody (lower panel). CBL- and CBLB-specific bands are not altered by immunodepletion of CBLB and CBL, respectively. B, lysates from 1.5 × 10^6 JEA2 cells were run on a polyacrylamide gel in quadruplicate. Each lane was excised and immunoblotted with anti-CBLB (lanes 1 and 2) or anti-CBL (lanes 3 and 4) antibodies in the presence of CBLB-specific (lanes 1 and 3) or irrelevant (lanes 2 and 4) peptide. CBLB-specific, but not CBL-specific, signal is inhibited by CBLB peptide.

FIG. 3. Analysis of CBLB phosphorylation in unstimulated and FL-stimulated JEA2 pro-B and THP1 monocytic cell lines. 5 × 10^6 THP1 (lanes 1 and 2) or JEA2 cells (lanes 3 and 4) were left unstimulated (lanes labeled −) or were stimulated for 5 min with 100 units/ml FL (lanes labeled +) and lysed in 1% Nonidet P-40-containing buffer. Lysates were then subjected to immunoprecipitation with anti-CBLB rabbit antiserum. Immunoprecipitates were separated by SDS-PAGE, transferred to nylon membrane, and subsequently immunoblotted with anti-phosphotyrosine (upper panel) or anti-CBL (lower panel). As controls, total cellular lysates of 5 × 10^6 nonstimulated THP1 (lane 5) or JEA2 (lane 6) cells were run along with the immunoprecipitates.

FIG. 4. Time-course analysis of FL-induced CBLB phosphorylation and association with p85PI3K, SHC, and GRB2. 15 × 10^6 JEA2 cells were left unstimulated or were stimulated for the indicated time with 100 units/ml FL and then lysed in 1% Nonidet P-40-containing buffer. Lysates were then subjected to immunoprecipitation with anti-CBLB goat antiserum. Immunoprecipitates were separated by SDS-PAGE, transferred to nylon membrane, and subsequently immunoblotted with the indicated antibodies. As controls, total cellular lysate of 1.5 × 10^6 nonstimulated JEA2 cells was run along with the immunoprecipitates. PY, phosphotyrosine.
CBL and CBLB do not associate with SOS1 in vivo despite a quantitatively similar GRB2 content in anti-CBL, anti-CBLB, and anti-SOS1 immunoprecipitates. This confirms that SOS1 and CBL are prominently present in distinct GRB2-containing complexes and extends this observation to CBLB. This indicates that CBL and CBLB may together regulate the RAS pathway by competing SOS1 for binding to GRB2.

CBL is a major phosphatidylinositol 3'-kinase-associated cytoplasmic protein, for example in cells stimulated by epidermal growth factor (27). The YEAM site was recently demonstrated to mediate phosphatidylinositol 3'-kinase binding (44). Surprisingly, this site is not conserved in CBLB. In consequence, CBLB binding to p85PI3K might be indirect or mostly SH3-dependent. As CBLB is tyrosine-phosphorylated, at least in response to FLT3 and IL-7 receptor activation, molecules that inducibly and directly bind CBLB through SH2-dependent interactions remain to be found.

A number of previous reports have demonstrated GRB2-mediated SHC binding to CBL (20, 38, 45). We demonstrate here that tyrosine-phosphorylated p52SHC also binds CBLB, probably via GRB2 as well. The functional significance of the recruitment of SHC to CBL and CBLB is presently unknown.

In view of their similarity with Sli1 (46) and D-CBL (47), which act as negative regulators of epidermal growth factor receptor-mediated transduction pathways in Caenorhabditis elegans and Drosophila melanogaster, respectively, CBL and CBLB may act to terminate proliferation induced by various growth factors and cytokines. However, only the N-terminal portion including the RING finger, is present in Sli1 and D-CBL, raising the possibility that CBL and CBLB have retained a functional protein-protein interaction domain, but may have different, if not divergent biological functions. In mammalian cells, recent reports have shown that CBL can behave as an effector (41) or a repressor (48) of tyrosine-dependent signaling pathways. This underlines the multifunctionality of CBL and possibly of CBLB as well.

Because of CBLB, together with CBL, is expressed in PBL and leukemic cells of lymphoid and myeloid origins, it is probably involved in a large number of tyrosine-kinase-regulated signaling processes in hematopoietic cells. We believe that tightly balanced CBL versus CBLB levels and/or compartmentalization may have a biological significance.

In conclusion, we have demonstrated for the first time that CBLB, like CBL, is a substrate for activated tyrosine kinase(s) and can bind to GRB2 and p52SHC, in addition to a number of...
tyrosine-phosphorylated proteins, including SHC. It is likely that CBLB participates in tyrosine kinase-regulated pathways in hematopoietic progenitors and more mature cells of the lympho-hematopoietic system.

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