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The Product of the *cbl* Oncogene Forms Stable Complexes In Vivo with Endogenous Crk in a Tyrosine Phosphorylation-Dependent Manner

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The cellular homologs of the v-Crk oncogene product are composed exclusively of Src homology region 2 (SH2) and SH3 domains. v-Crk overexpression in fibroblasts causes cell transformation and elevated tyrosine phosphorylation of specific cellular proteins. Among these proteins is a 130-kDa protein, identified as p130^{cas}, that forms a stable complex in vivo with v-Crk. We have explored the role of endogenous Crk proteins in Bcr-Abl-transformed cells. In the K562 human chronic myelogenous leukemia cell line, p130^{cas} is not tyrosine phosphorylated or bound to Crk. Instead, Crk proteins predominantly associate with the tyrosine-phosphorylated proto-oncogene product of Cbl. In vitro analysis showed that this interaction is mediated by the SH2 domain of Crk and can be inhibited with a phosphopeptide containing the Crk-SH2 binding motif. In NIH 3T3 cells transformed by Bcr-Abl, c-Cbl becomes strongly tyrosine phosphorylated and associates with c-Crk. The complex between c-Crk and c-Cbl is also seen upon T-cell receptor cross-linking or with the transforming, tyrosine-phosphorylated c-Cbl. These results indicate that Crk binds to c-Cbl in a tyrosine phosphorylation-dependent manner, suggesting a physiological role for the Crk–c-Cbl complex in Bcr-Abl tyrosine phosphorylation-mediated transformation.

The Crk proteins, originally isolated as an oncogene product in an avian sarcoma virus (26), belong to a class of adapter proteins containing Src homology 2 (SH2) and SH3 domains. Other members of this group include Grb2/ASH (11) and Nck (13). These bifunctional proteins are thought to couple tyrosine-phosphorylated receptors or their substrates via the SH2 domain to downstream effectors via the SH3 domain. Three cellular homologs of v-Crk have been identified: Crk-I, a 21-kDa protein with only one SH2 and one SH3 domain (24); Crk-II, 40- and 42-kDa proteins consisting of one SH2 and two SH3 domains (36); and CrkL, a 36-kDa Crk-like protein with one SH2 and two SH3 domains (49). Despite their lack of demonstrated enzymatic activity, expression of v-Crk or Crk-I but not Crk-II leads to cell transformation and increased tyrosine phosphorylation (24, 27). Furthermore, v-Crk binds directly to the major tyrosine-phosphorylated proteins present in v-Crk-transformed cells, presumably via its SH2 domain (22, 23). These phosphoproteins include paxillin, a focal adhesion-associated protein (3), and the newly identified p130^{cas} (39). p130^{cas} has no known catalytic domain but contains a cluster of predicted high-affinity Crk-SH2 binding motifs and forms a stable complex in vivo with v-Crk. The Crk-SH3 domain has been shown to interact with several proteins, including Abl (8), Sos (9, 21), C3G (9, 15, 21, 46), and, more recently, Eps15 (42). Since Sos and C3G contain a guanine nucleotide exchange activity, Crk proteins have been hypothesized to play a role in the regulation of p21^{ras}-GTP formation. Both the SH2 and SH3 domains of v-Crk are required for the transformation of chicken embryo fibroblasts (27). In addition, Crk mutants with dysfunctional SH2 or SH3 domains inhibited neuronal differentiation of PC-12 cells and nerve growth factor-induced activation of Ras (21, 45).

The c-Abl oncogene product is a member of the nonreceptor class of tyrosine kinases. Like c-Crk-II, c-Abl overexpression does not lead to cellular transformation (10, 14). However, the chromosomal translocation of c-*abl* to the *bcr* gene in Philadelphia chromosome-positive human leukemias produced the 210- and 190-kDa chimeric Bcr-Abl proteins (6, 16). These fusion proteins have elevated tyrosine kinase activity and transforming properties (20, 32). Although the precise mechanisms involved in cellular transformation by Bcr-Abl remain unclear, several key signaling proteins are known to be tyrosine phosphorylated in cells bearing this oncogene, including Bcr-Abl itself (32) as well as Shc (35), CrkL (48), paxillin (40), and the proto-oncogene product of *cbl* (1). Moreover, activation of Ras has also been implicated in Bcr-Abl-mediated cellular transformation (34, 35).

To evaluate the potential role of Crk proteins in the transforming functions of Bcr-Abl, we have examined their interactions in the human chronic myelogenous leukemia cell line K562. We report here that, unlike observations in v-Crk- and v-Src-transformed cells, p130^{cas} is not tyrosine phosphorylated or bound to Crk in K562 cells. Instead, Crk proteins associate predominantly with the tyrosine-phosphorylated c-Cbl proto-oncogene product through its SH2 domain. These results suggest that the Crk–c-Cbl complex may play a role in promoting the tumorigenic process initiated by Bcr-Abl tyrosine kinase.

MATERIALS AND METHODS

Cell cultures. The Philadelphia chromosome-positive leukemic K562 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1% penicillin-streptomycin. The murine hematopoietic cell line 70Z/3 was obtained from the American Type Culture Collection (Rockville, Md.). 70Z/3 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 1% penicillin-streptomycin. The Jurkat T-cell line and JY normal human B cells

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were a generous gift from L. Petruzzelli (University of Michigan, Ann Arbor). The Jurkat T cells were maintained and stimulated by anti-CD3ε monoclonal antibody OKT3 as described before (7). Eighteen hours prior to activation, the Jurkat T cells were placed in serum-free RPMI 1640 medium. Bcr-Abl-transformed NIH 3T3 cells were a generous gift from Jean Y. J. Wang (37). Normal and transformed NIH 3T3 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 2% penicillin-streptomycin.

Immunoprecipitations and immunoblotting. Cells (0.5×10^7 to 1×10^7 cells per sample) were washed twice with ice-cold phosphate-buffered saline before the addition of lysis buffer containing 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM sodium PP_i, 1 mM sodium orthovanadate, 100 mM NaF, 10 μg of each aprotenin and leupeptin per ml, and 1 mM phenylmethylsulfonyl fluoride and incubated on ice for 15 min. After centrifugation at $10,000 \times g$ for 15 min at 4°C, proteins were immunoprecipitated with either 2 μg of anti-c-Crk-II, anti-p130^{cas} (Transduction Labs, Lexington, Ky.), anti-c-Cbl, anti-Crk-L (Santa Cruz Inc.), or anti-Bcr (Oncogene Science) antibodies. After 12 h at 4°C, immunoprecipitates were mixed with protein G- or protein A-agarose (Oncogene Science) for 2 h and washed with lysis buffer, and the immunocomplexes were solubilized at 100°C for 5 min in 25 μl of Laemmli sample buffer. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. Individual proteins were detected with the specified antibodies, and phosphotyrosine was detected with a mixture of the antiphosphotyrosine antibodies RC20H (Transduction Labs) and 4G10 (Upstate Biotechnology, Inc., Lake Placid, N.Y.). Bound antibodies were detected with horseradish peroxidase-linked secondary antibodies with the enhanced chemiluminescence system (ECL; Amersham Corp.). To reprobe the immunoblots, the nitrocellulose membranes were incubated for 30 min at 60°C with 62.5 mM Tris-HCl (pH 6.8) containing 2% SDS and 0.7% 2-mercaptoethanol and then washed extensively with 10 mM Tris-HCl (pH 8)–150 mM NaCl.

In vitro binding assays and phosphopeptide competition. The glutathione-*S*-transferase (GST) fusion proteins containing the SH2 and SH3 domains of Crk were a generous gift of R. B. Birge and H. Hanafusa (3, 9). The GST-Grb2 fusion protein has been described previously (25), and the generation and production of the GST-Grb2-SH2 fusion protein was done as described elsewhere (19). For in vitro association experiments, GST fusion proteins bound to glutathione-agarose beads or GST alone was incubated for 90 min at 4°C with 250 μl of cell lysate (equivalent to 10^7 cells in lysis buffer). After the beads were washed three times with lysis buffer, the adsorbed proteins were eluted and analyzed as described above for immunoprecipitates. The phosphotyrosine-containing peptides derived from the major phosphorylation sites of the epidermal growth factor (EGF) receptor, DADEpYLIPQQG (pY992) and VPEpYINQSVPK (pY1068), were synthesized, purified, and characterized as described previously (29). For phosphopeptide competition experiments, peptides (final concentration, 300 μM) were incubated with the GST-SH2 fusion proteins for 30 min prior to addition of cell lysates, and the incubation was continued for 90 min. After being washed with lysis buffer, the SH2-bound proteins were separated by SDS-PAGE and identified by immunoblotting with antiphosphotyrosine or anti-c-Cbl antibodies.

RESULTS

p130^{cas} is not tyrosine phosphorylated in K562 cells. In v-Crk- and v-Src-transformed cells, the major tyrosine-phosphorylated 130-kDa protein was recently identified as p130^{cas} (39). p130^{cas} associates directly with v-Crk and v-Src in these cells, suggesting that tyrosine phosphorylation of p130^{cas} may play an important role in cellular transformation by these oncoproteins (39). Both c-Crk-II and CrkL have been shown to bind to c-Abl (8, 48), and CrkL is phosphorylated on tyrosine in the human chronic myelogenous leukemia cell line K562, presumably by activated Bcr-Abl tyrosine kinase (48). Therefore, we were interested to examine the possibility that p130^{cas} is involved in Bcr-Abl-mediated tyrosine phosphorylation and associated with endogenous Crk in K562 cells. Lysates were prepared and incubated with anti-Bcr, anti-p130^{cas}, or anti-c-Crk-II antibodies, and the precipitated proteins were analyzed by immunoblotting with antiphosphotyrosine antibodies (Fig. 1A). In the anti-c-Crk-II immunoprecipitates, a phosphotyrosine-containing protein with an apparent molecular mass of 120 kDa was detected. However, the anti-p130^{cas} immunoprecipitate did not contain any tyrosine-phosphorylated proteins, suggesting that the 120-kDa c-Crk-II-associated protein is not p130^{cas}. With anti-Bcr antibodies, a phosphotyrosine-containing protein with a molecular mass of 210 kDa that has been shown to be the product of the *bcr-abl* gene was immunoprecipitated (20, 32). The blot shown in Fig. 1A was stripped and reprobed with anti-p130^{cas} antibodies to confirm the presence of p130^{cas} in K562 cells (Fig. 1B). Immunoreactive p130^{cas} protein was detected only in the anti-p130^{cas} antiserum immunoprecipitate. These results demonstrate that tyrosine phosphorylation of p130^{cas} is not involved in the cellular transformation induced by the activated Bcr-Abl tyrosine kinase.

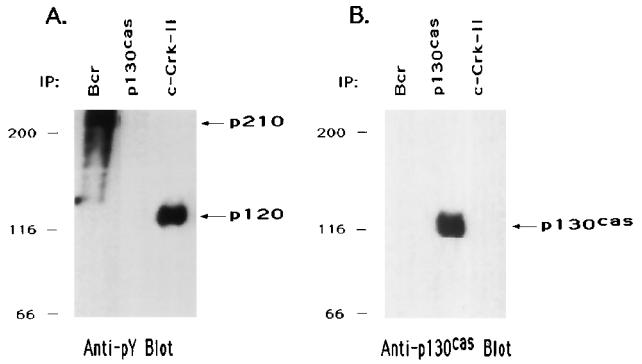

FIG. 1. p130^{cas} is not tyrosine phosphorylated or bound to c-Crk-II in K562 cells. (A) K562 cell lysates (10^7 cells per sample) were immunoprecipitated (IP) with anti-Bcr, anti-p130^{cas}, or anti-c-Crk-II antibodies. The resulting immunoprecipitates were separated by SDS-PAGE and subjected to immunoblotting with antiphosphotyrosine (anti-pY) antibodies. (B) The immunoblot shown in panel A was stripped and reprobed with anti-p130^{cas} antibodies. The blots were visualized by chemiluminescence. The positions of 210-kDa Bcr-Abl (p210), 120-kDa protein (p120) and p130^{cas} are indicated by arrows. The positions of molecular mass markers are shown at the left (in kilodaltons).

Identification of p120 c-Crk-II-associated protein as the *cbl* proto-oncogene product in K562 cells. Recently, it has been shown that the proto-oncogene product of *cbl* is tyrosine phosphorylated in tumor cells expressing v-Abl or Bcr-Abl tyrosine kinases (1). The presence of a 120-kDa tyrosine-phosphorylated protein in the anti-c-Crk-II immunoprecipitates (Fig. 1A) led us to examine whether this protein might be c-Cbl. K562 cell lysates were incubated with either anti-c-Crk-II or anti-c-Cbl antibodies, followed by immunoblotting with antiphosphotyrosine, anti-c-Cbl, or anti-c-Crk-II antibodies. As shown in Fig. 2A, the 120-kDa c-Crk-II-associated phosphotyrosine-containing protein comigrated with a tyrosine-phosphorylated band of approximately equal intensity present in the anti-c-Cbl immunoprecipitates. In both the anti-c-Cbl and anti-c-Crk-II immunoprecipitates, the 120-kDa tyrosine-phosphorylated protein immunoreacted with the anti-c-Cbl antibodies (Fig. 2B). This result indicates that c-Crk-II forms a stable complex in vivo with c-Cbl in K562 cells. Quantitation of the immunoblot shown in Fig. 2B revealed that the anti-c-Crk-II antibodies immunoprecipitated 70 to 80% of the pool of c-Cbl present in the anti-c-Cbl immunoprecipitates. The 40-kDa tyrosine-phosphorylated protein in the anti-c-Crk-II precipitate was identified as c-Crk-II by immunoblotting with anti-c-Crk-II antibodies (Fig. 2C). However, we were unable to detect c-Crk-II in the anti-c-Cbl immunoprecipitates under these conditions.

SH2 domain of Crk binds to the tyrosine-phosphorylated c-Cbl. Experiments carried out with peptide libraries have shown that the SH2 domain of Crk specifically binds to phosphotyrosine-containing peptides with the primary sequence Yp-X-X-P (44). Three such motifs appear in c-Cbl (5). The Crk N-terminal SH3 domain has been shown to interact with Abl (8), Sos (9, 21), C3G (9, 15, 21, 46), and recently with Eps15 (42). To identify which Crk domain(s) binds to c-Cbl, we incubated K562 cell lysates with the SH2 and the N-terminal

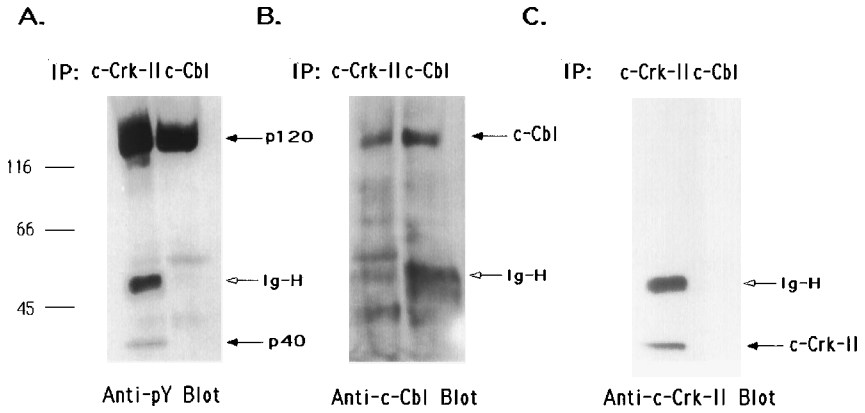

FIG. 2. c-Cbl is the 120-kDa c-Crk-II-associated phosphoprotein in K562 cells. Lysates from K562 cells (10^7 cells per sample) were immunoprecipitated (IP) with anti-c-Crk-II or anti-c-Cbl antibodies. Following electrophoresis and transfer to nitrocellulose, samples were immunoblotted with antiphosphotyrosine (anti-pY) antibodies (A), anti-c-Cbl antibodies (B), or anti-c-Crk-II antibodies (C) and detected by chemiluminescence. The positions of 120-kDa (p120) and 40-kDa (p40) proteins (A), c-Cbl (B), and c-Crk-II (C) are indicated by arrows. Ig-H, immunoglobulin heavy chain. The positions of molecular mass markers are indicated at the left (in kilodaltons).

SH3 [Crk-SH3(N)] domains of Crk expressed as GST fusion proteins immobilized on glutathione-agarose beads. The bound proteins were separated by SDS-PAGE and detected by immunoblotting with antiphosphotyrosine or anti-c-Cbl antibodies. As shown in Fig. 3A, the different fusion proteins precipitated a set of specific tyrosine-phosphorylated proteins. The GST-Crk-SH2, GST-Crk-SH3(N), and full-length GST-Grb2 fusion proteins but not GST alone precipitated 210- and 145-kDa tyrosine-phosphorylated proteins, shown to be the Bcr-Abl and c-Abl proteins, respectively (data not shown) (8, 34, 48). The GST-Grb2-SH2 fusion protein precipitated only the 210-kDa Bcr-Abl protein (34). Additionally, both GST-Grb2 and GST-Grb2-SH2 precipitated a tyrosine-phosphorylated protein of 52 kDa, previously identified as Shc (18).

The 120-kDa phosphotyrosine-containing protein was detected in GST-Crk-SH2 and GST-Grb2 precipitates but not with GST-Crk-SH3(N), GST-Grb2-SH2, or GST alone. To test whether this protein is c-Cbl, the same blot was stripped and reprobed with anti-c-Cbl antibodies. Figure 3B shows that c-Cbl comigrated on SDS-PAGE with the 120-kDa phosphotyrosine-containing protein precipitated by GST-Crk-SH2 and GST-Grb2. This experiment also demonstrates that GST-Crk-SH3(N) does not bind to the phosphorylated or nonphosphorylated form of c-Cbl. The interaction of c-Cbl with GST-Grb2 and not with GST-Grb2-SH2 is in agreement with previous studies, carried out in T cells, that showed constitutive association of c-Cbl with Grb2 through its SH3 domain (7). These results demonstrate that Crk binds to c-Cbl exclusively through its SH2 domain.

To further explore the specificity of GST-Crk-SH2 binding to these phosphoproteins, we assessed the ability of phosphopeptides derived from two of the major autophosphorylation sites of the EGF receptor (Y-992 and Y-1068) to disrupt the GST-Crk-SH2 interactions with the above-described tyrosine-phosphorylated proteins. GST-Crk-SH2 and GST-Grb2-SH2 were incubated with the phosphopeptides prior to the addition of K562 cell lysates. As evident from the antiphosphotyrosine immunoblotting of the bound proteins (Fig. 3C), binding of Bcr-Abl, c-Abl, and c-Cbl to GST-Crk-SH2 was quantitatively inhibited by the phosphopeptide DADEpYLI PQQG (pY-992), which contains a proline in the +3 position relative to tyrosine, a sequence preferentially recognized by the Crk SH2 domain. The phosphopeptide VPEpYINQSVPK (pY-1068) had little effect on proteins binding to GST-Crk-SH2 under the same conditions. Reprobing the same immunoblot with anti-c-Cbl antibodies revealed a concordant decrease in the amount of c-Cbl associated with the Crk SH2 domain in the presence of pY-992 (Fig. 3D).

The SH2 region of Grb2 is predicted to bind to the consensus sequence Yp-X-N-Y (44). The high-affinity binding site for Grb2 on the EGF receptor has been mapped to the autophosphorylation site Y-1068 (2). The phosphotyrosine peptide pY-1068, which contains the Grb2 SH2 recognition site, markedly reduced the binding of GST-Grb2-SH2 to the tyrosine-phosphorylated 52-kDa Shc proteins. However, the pY-992 peptide, which does not contain this motif, had no effect on GST-Grb2-SH2 binding (Fig. 3E).

CrkL forms complexes with c-Cbl and Bcr-Abl in K562 cells. The Crk-like protein CrkL, a 36-kDa protein recently cloned from K562 cells (49), is composed of one SH2 domain and two SH3 domains. This protein is similar to the c-Crk-II protein in its amino acid sequences in the SH2 and SH3 domains. CrkL has been shown to be tyrosine phosphorylated and associated with Bcr-Abl in K562 cells (48). To further characterize the interaction between c-Cbl and CrkL, K562 cell lysates were immunoprecipitated with anti-Bcr, anti-c-Cbl, or anti-CrkL antibodies followed by immunoblotting with antiphosphotyrosine antibodies (Fig. 4). Both anti-c-Cbl and anti-CrkL antibodies immunoprecipitated a 120-kDa phosphotyrosine-containing protein confirmed to be c-Cbl (Fig. 4A). In addition to the tyrosine-phosphorylated CrkL protein (Fig. 4B), the anti-CrkL antibodies also precipitated a 210-kDa tyrosine-phosphorylated protein that was confirmed to be Bcr-Abl, as previously reported (48). The similar association of c-Crk-II with Bcr-Abl could not be detected in the anti-c-Crk-II immunoprecipitates (Fig. 1A).

Transformation by Bcr-Abl induces tyrosine phosphorylation of c-Cbl and the formation of a c-Crk–c-Cbl complex. To determine if c-Crk–c-Cbl association in K562 cells is due to activated Bcr-Abl tyrosine kinase, we further analyzed their interaction in NIH 3T3 fibroblasts transformed by expression of the *bcr-abl* gene. Cell lysates prepared from normal NIH 3T3 fibroblasts and NIH 3T3 cells transformed by Bcr-Abl were immunoprecipitated with anti-Bcr or anti-c-Cbl antibodies, followed by immunoblotting with antiphosphotyrosine or anti-c-Cbl antibodies (Fig. 5A). Tyrosine-phosphorylated 190-

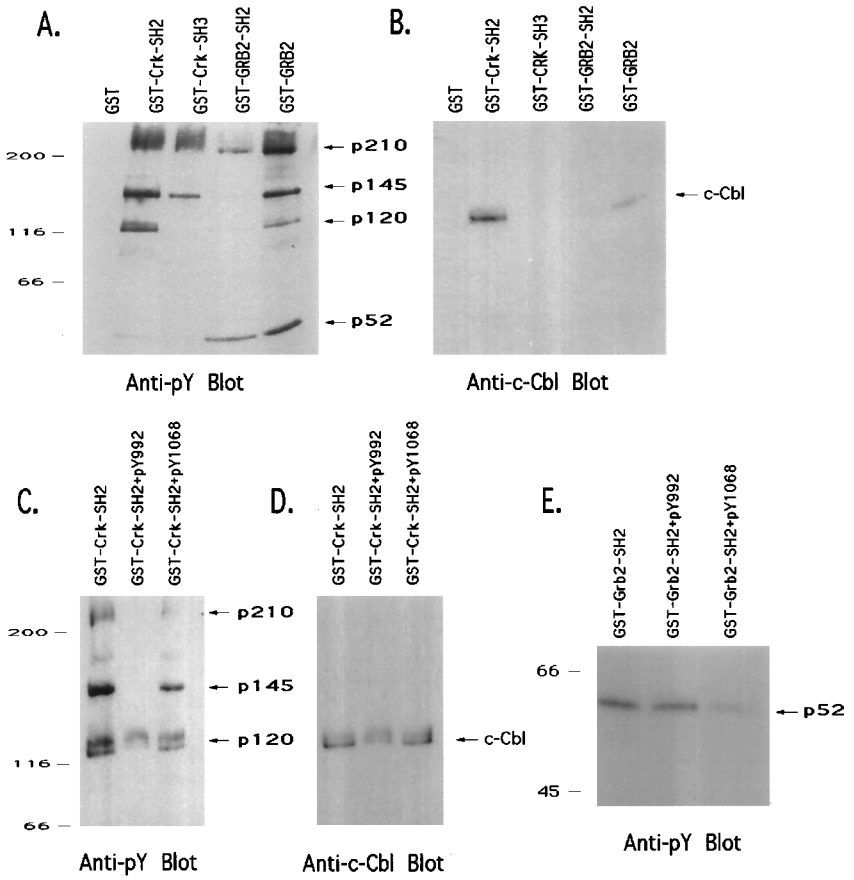

FIG. 3. SH2 domain of Crk binds to the tyrosine-phosphorylated c-Cbl. (A) K562 cell lysates were incubated with GST-Crk-SH2, GST-Crk-SH3(N), GST-full-length Grb2, or GST-Grb2-SH2 fusion protein or GST alone immobilized on glutathione-agarose beads for 90 min at 4°C. Bound proteins were eluted and separated by SDS-PAGE followed by immunoblotting with antiphosphotyrosine (anti-pY) antibodies. (B) The blot from panel A was stripped and reprobed with anti-c-Cbl antibodies. For the phosphopeptide competition, the fusion proteins GST-Crk-SH2 and GST-Grb2-SH2 were incubated in the absence or in the presence of phosphopeptide DADEpYLIPQQG (pY-992) or VPEpYINQSVPK (pY-1068) as described in Materials and Methods. Bound proteins were separated by SDS-PAGE followed by immunoblotting with antiphosphotyrosine (C and E). The blot from panel C was stripped and reprobed with anti-c-Cbl antibodies (D). The positions of 210-kDa (Bcr-Abl, p210), 145-kDa (Abl, p145), 120-kDa (p120), c-Cbl, and 52-kDa (Shc, p52) proteins are indicated as appropriate. The positions of molecular mass markers are indicated at the left (in kilodaltons). The blots were visualized by chemiluminescence.

and 210-kDa proteins, identified as the Bcr-Abl tyrosine kinase, were detected in the anti-Bcr immunoprecipitates from the NIH 3T3 fibroblasts transformed by Bcr-Abl (37). As can be seen in the antiphosphotyrosine immunoblot (Fig. 5A, anti-pY), c-Cbl in normal NIH 3T3 fibroblasts has no detectable phosphotyrosine. In contrast, c-Cbl is heavily tyrosine phosphorylated in Bcr-Abl-transformed fibroblasts. In addition, c-Cbl precipitates from NIH 3T3 fibroblasts transformed by Bcr-Abl also contain a 210-kDa tyrosine-phosphorylated protein identified as Bcr-Abl. The level of c-Cbl does not appear to differ in the two cell lines (Fig. 5A, anti-c-Cbl). To test for c-Crk–c-Cbl complex formation, cell lysates prepared from normal NIH 3T3 fibroblasts or fibroblasts transformed by Bcr-Abl were precipitated with anti-c-Cbl or anti-c-Crk-II antibodies and the GST fusion proteins with Crk-SH3(N) and Crk-SH2. The bound proteins were then analyzed by immunoblotting with anti-c-Cbl antibodies (Fig. 5B). c-Cbl could be detected in the anti-c-Crk and GST-Crk-SH2 precipitates from Bcr-Abl-transformed NIH 3T3 cells. In normal NIH 3T3 cells, c-Cbl was not detected in the anti-c-Crk immunoprecipitates. These results demonstrate that binding of c-Cbl to c-Crk is dependent on Bcr-Abl tyrosine kinase-induced phosphorylation of c-Cbl.

Association of c-Cbl with c-Crk-II in anti-CD3-stimulated Jurkat T cells. We next examined whether the interaction between tyrosine-phosphorylated c-Cbl and c-Crk depends exclusively on the presence of activated Bcr-Abl tyrosine kinase or whether it could also be induced in other tyrosine kinase signaling pathways. Recent studies have shown that in hematopoietic cells, c-Cbl is constitutively associated with the SH3 domain of Grb2 and undergoes tyrosine phosphorylation upon cell stimulation (7, 33). The association between c-Cbl and c-Crk was further investigated in Jurkat T cells following stimulation by cross-linking of the T-cell antigen receptor (TCR) with OKT3. Lysates from serum-starved and OKT3-activated Jurkat T cells were incubated with anti-c-Cbl or anti-c-Crk-II antibodies, the fusion proteins GST-Crk-SH2, GST-Crk-SH3(N), GST-Grb2-SH2, and full-length Grb2 or GST alone. The resulting precipitates were separated by SDS-PAGE and blotted with antiphosphotyrosine antibodies (Fig. 6). In unstimulated cells, a low level of tyrosine phosphorylation was identified in a 120-kDa protein that was precipitated with anti-c-Cbl and anti-c-Crk-II antibodies as well as with the GST fusion proteins containing Crk SH2 or full-length Grb2. TCR cross-linking resulted in increased tyrosine phosphorylation of the 120-kDa protein precipitated with the anti-c-Cbl and anti-

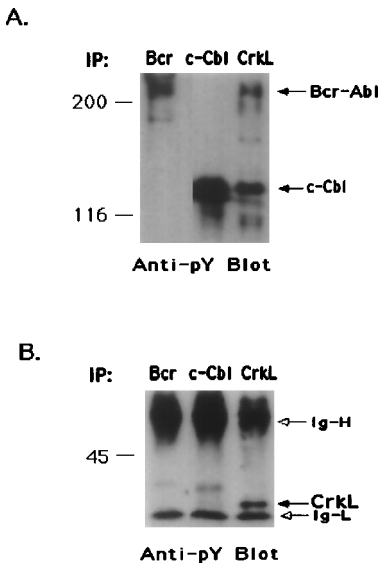

FIG. 4. CrkL forms a complex with Bcr-Abl and c-Cbl in K562 cells. Lysates prepared from K562 cells were immunoprecipitated (IP) with anti-Bcr, anti-c-Cbl, or anti-CrkL antibodies. Bound proteins were resolved and analyzed by immunoblotting with antiphosphotyrosine (anti-pY) antibodies. Panel B is a longer exposure of the blot in panel A. The blots were visualized by chemiluminescence. The positions of c-Cbl (A) and CrkL (B) are indicated by arrows. Ig-H and Ig-L, immunoglobulin heavy and light chains, respectively. The positions of molecular mass markers are indicated at the left (in kilodaltons).

c-Crk-II antibodies and enhanced the association of this phosphoprotein with GST-Crk-SH2 and GST-Grb2. The 120-kDa phosphotyrosine-containing protein was not detected in precipitates of GST-Crk-SH3(N), GST-Grb2-SH2, or GST alone. When the membrane used for this experiment was stripped of the antibodies and reprobed with the anti-c-Cbl antiserum, we found that c-Cbl protein comigrated with the 120-kDa tyrosine-phosphorylated protein, consistent with previous reports (data not shown) (7, 33). TCR activation increased the amount of c-Cbl associated with endogenous c-Crk and bound by the Crk SH2 domain fusion protein, supporting the notion that the tyrosine-phosphorylated form of c-Cbl associates with c-Crk through its SH2 domain. c-Cbl was not present in precipitates of GST-Grb2-SH2 or GST alone in either nonactivated or activated Jurkat T cells.

As previously reported and shown in Fig. 6 (31, 43), we have observed a 36-kDa tyrosine-phosphorylated protein associated with Grb2 and its SH2 domain after TCR stimulation. However, we detected only very low levels of a 36-kDa phosphoprotein bound to the Crk SH2 domain fusion protein following activation of Jurkat T cells.

Association of c-Crk-II with oncogenic form of c-Cbl. The mutations in c-Cbl that promote transformation were also found to induce tyrosine phosphorylation of c-Cbl. In the pre-B-lymphoma 70Z/3 cell line, a mutant form of c-Cbl that carries a deletion from amino acids 366 to 382 became tyrosine phosphorylated and induced transformation (1). The deleted sequences do not include the Crk SH2 binding motifs in c-Cbl. To determine if this oncogenic form of c-Cbl associates with c-Crk, 70Z/3 cell lysates were precipitated with anti-c-Cbl or anti-c-Crk-II antibodies and the fusion proteins GST-Crk-SH3(N), GST-Crk-SH2, and GST-Grb2-SH2 or GST alone. The bound proteins were resolved by SDS-PAGE, followed by immunoblotting with anti-c-Cbl antibodies (Fig. 7A). As shown above for the K562 cell line, c-Cbl coimmunoprecipitated with anti-c-Cbl or anti-c-Crk-II antibodies and the fusion protein GST-Crk-SH2 but not with GST-Crk-SH3(N), GST-Grb2-SH2, or GST alone. In 70Z/3 cells, only c-Cbl protein translated from the mutant allele is tyrosine phosphorylated (1). In contrast, c-Cbl is not tyrosine phosphorylated in JY normal human B cells and does not associate with c-Crk (Fig. 7B), although these cells express detectable amounts of c-Cbl (Fig. 7C). These results further demonstrate the tyrosine phosphorylation-dependent interaction of c-Cbl with endogenous Crk.

DISCUSSION

Cellular overexpression of the oncogenic form of Crk leads to cell transformation and elevation of intracellular tyrosine phosphorylation levels of specific proteins (3, 22, 24, 26, 27, 39). Although this protein apparently lacks catalytic activity, both its transforming and differentiating functions require intact SH2 and SH3 domains (27, 45). v-Crk binds directly to the phosphotyrosine-containing proteins in v-Crk-transformed cells, presumably via its SH2 domain (3, 22, 23, 27, 39). Crk has also been shown to associate with two guanine nucleotide exchange proteins, Sos and C3G, via its SH3 domain (9, 15, 21, 46), suggesting a role in regulating the Ras signaling pathway. The major tyrosine-phosphorylated protein detected in fibro-

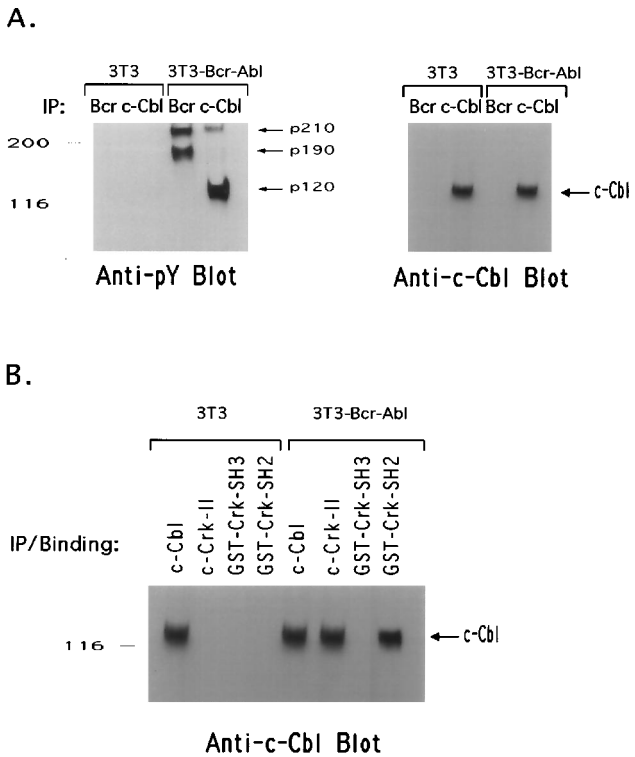

FIG. 5. c-Cbl is tyrosine phosphorylated and associates with c-Crk-II in fibroblasts transformed by Bcr-Abl. (A) Lysates prepared from NIH 3T3 fibroblasts (3T3) or NIH 3T3 fibroblasts expressing Bcr-Abl (3T3 Bcr-Abl) were immunoprecipitated (IP) with anti-Bcr or anti-c-Cbl antibodies. The bound proteins were resolved by SDS-PAGE followed by immunoblotting with antiphosphotyrosine (anti-pY) or anti-c-Cbl antibodies. (B) Lysates prepared from NIH 3T3 fibroblasts (3T3) or NIH 3T3 fibroblasts expressing Bcr-Abl (3T3 Bcr-Abl) were precipitated (IP/Binding) with anti-c-Cbl or anti-c-Crk-II antibodies and the fusion proteins GST-Crk-SH3(N) and GST-Crk-SH2. The resulting precipitates were subjected to immunoblotting with anti-c-Cbl antibodies. The positions of 210- and 190-kDa (Bcr-Abl; p210 and p190) and 120-kDa (p120) proteins and c-Cbl are indicated by arrows. The positions of molecular mass markers are indicated at the left (in kilodaltons). The blots were visualized by chemiluminescence.

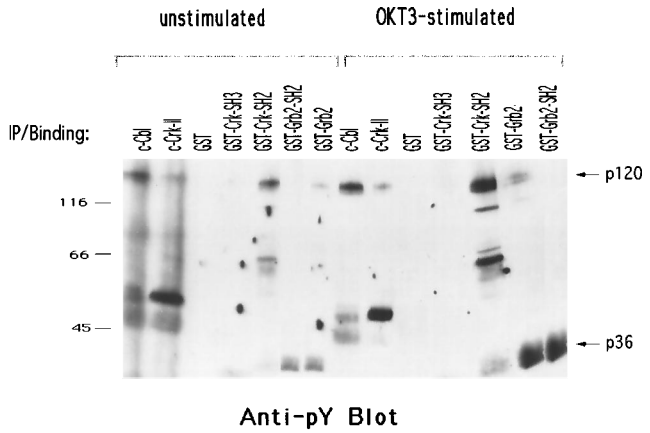

FIG. 6. c-Crk-II and GST-Crk-SH2 associate with c-Cbl upon stimulation of Jurkat T cells. Serum-starved Jurkat T cells (2×10^7 cells per sample) were either unstimulated or stimulated by OKT3-mediated cross-linking. Cell lysates were prepared and precipitated (IP/Binding) with anti-c-Cbl or anti-c-Crk-II antibodies and the fusion proteins GST-Crk-SH3(N), GST-Crk-SH2, GST-Grb2-SH2, and full-length Grb2 or GST alone. The resulting precipitates were subjected to immunoblotting with anti-phosphotyrosine (anti-pY) antibodies and visualized by chemiluminescence. The arrows indicate the positions of 120-kDa (p120) and 36-kDa (p36) proteins. The positions of molecular mass markers are indicated at the left (in kilodaltons).

blasts transformed by v-Crk as well as by the nonreceptor tyrosine kinase v-Src has recently been cloned (39). This 130-kDa protein, called Cas, associates directly with v-Crk in these cells (39). p130^{cas} is apparently devoid of kinase activity but contains clusters of tyrosine phosphorylation sites with a proline in the +3 position, suggesting its specific interaction with the Crk SH2 domain.

Chronic myelogenous leukemia and Philadelphia chromosome-positive acute leukemia are characterized by a specific chromosomal translocation that results in the fusion of Bcr-encoded sequences upstream of c-Abl (6, 16). The chimeric Bcr-Abl protein has deregulated kinase activity and transforming potential (20, 32). Recent data indicated that the c-Abl kinase binds first to the Crk SH3 domain and can phosphorylate Tyr-221 in the spacer region between the two SH3 domains in Crk (8). In chronic lymphoblastic leukemia, the c-Crk-II homolog CrkL is constitutively tyrosine phosphorylated and associates in vivo with Bcr-Abl protein (48).

In an effort to identify the role of Crk in the prevention of apoptosis in transformed hematopoietic cell lines, we observed that p130^{cas} is not tyrosine phosphorylated in chronic lymphoblastic leukemia K562 cells expressing activated Bcr-Abl kinase, although we observed that both forms of Crk, c-Crk-II and CrkL, are constitutively tyrosine phosphorylated in these cells. It has been shown recently that c-Abl phosphorylates p130^{cas} in vitro (28). However, our result demonstrating the inability of Bcr-Abl to promote p130^{cas} tyrosine phosphorylation may indicate that p130^{cas} is not an endogenous target of the activated Bcr-Abl tyrosine kinase and thus is not involved in Bcr-Abl-mediated cellular transformation. Another tyrosine-phosphorylated protein identified in v-Crk-transformed cells is paxillin, a focal adhesion-associated protein (3). Recently it has been shown that paxillin is tyrosine phosphorylated in myeloid cell lines transformed by Bcr-Abl and coprecipitated with Bcr-Abl (40). We could not detect tyrosine-phosphorylated paxillin in K562 cells or its association with Crk (data not shown).

We observed a major phosphotyrosine-containing protein with a molecular mass of 120 kDa that associates with endogenous Crk proteins in K562 cells. We identified this protein as the product of the proto-oncogene *cbl* (5). In vitro binding studies showed that the association between c-Cbl and Crk is mediated by the SH2 domain of Crk. c-Cbl has three potential tyrosine phosphorylation motifs recognized by the Crk SH2 domain (5, 44). Although the in vitro binding of a GST-Crk-SH2 fusion protein to c-Cbl could be blocked by a phosphopeptide containing the Crk SH2 binding motif, it remains to be determined whether one or all three of the tyrosine phosphorylation motifs in c-Cbl participate in the association with Crk.

The c-*cbl* gene was cloned as the cellular homolog of the v-*cbl* oncogene, which is transforming in early B-lineage and myeloid cells (17). The viral oncogene product lacks 62% of the C-terminal sequences of the cellular homolog (5). The truncated protein encoded by v-*cbl* is localized in both the cytoplasm and the nucleus, where it can bind DNA, while the cellular form is cytoplasmic (4). While the primary structure of c-Cbl resembles that of DNA-binding transcription factors, with a nuclear localization sequence, a zinc finger-like motif, a leucine zipper, and a proline-rich domain, its physiological function has yet to be determined. Overexpression of c-Cbl does not induce transformation; however, mutations in c-Cbl that promote transformation were found to induce tyrosine phosphorylation of c-Cbl (1). Recently it has become apparent that c-Cbl becomes tyrosine phosphorylated in response to activation of a variety of tyrosine kinase signaling pathways (1, 7, 12, 33, 47), suggesting that it may be an important component of signal transduction downstream of tyrosine kinases. c-Cbl is constitutively associated with the adapters Grb2 and Nck through their SH3 domains (7, 33, 38). In contrast, our results demonstrate that the association of c-Cbl with c-Crk-II and CrkL is dependent on the tyrosine phosphorylation of

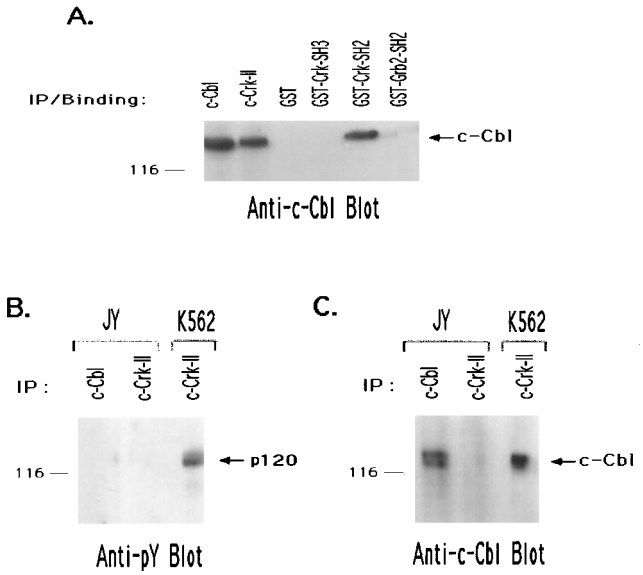

FIG. 7. Association of c-Crk-II with the tyrosine-phosphorylated oncogenic form of c-Cbl. (A) Lysates prepared from 70Z/3 cells carrying the mutant form of c-Cbl were precipitated with anti-c-Cbl or anti-c-Crk-II antibodies and the fusion proteins GST-Crk-SH3(N), GST-Crk-SH2, and GST-Grb2-SH2 or GST alone. The bound proteins were resolved by SDS-PAGE followed by immunoblotting with anti-c-Cbl antibodies. JY normal human B cells or K562 cells were lysed and immunoprecipitated with anti-c-Cbl or anti-c-Crk-II antibodies. The immunoprecipitates were subjected to immunoblot analysis with antiphosphotyrosine (anti-pY) (B) or anti-c-Cbl (C) antibodies. The positions of 120-kDa protein (p120) and c-Cbl are indicated by arrows. The positions of molecular mass markers are indicated at the left (in kilodaltons). The blots were visualized by chemiluminescence.

c-Cbl and mediated through the SH2 domain of Crk (Fig. 3). Upon T-cell activation, both c-Cbl and p116 bind to Crk proteins (Fig. 6) (41). In addition, c-Cbl has recently been shown to associate with the p85 subunit of phosphatidylinositol 3-kinase in activated T cells (30).

Although the precise significance of the c-Cbl-Crk interaction remains unknown, the association of these proteins correlates well with cellular transformation in Bcr-Abl- and oncogenic c-Cbl-expressing cells. Moreover, c-Cbl is the major Crk-associated tyrosine-phosphorylated protein detected in both cells, suggesting further the biological significance of this phenomenon. We also detected the association of c-Crk with tyrosine-phosphorylated c-Cbl in vivo upon TCR activation. Interestingly, the c-Cbl-Crk association was not detected in mouse 3T3-L1 cells stimulated with insulin or in EGF-treated NIH 3T3 cells overexpressing the EGF receptor despite the tyrosine phosphorylation of c-Cbl produced by both hormones (data not shown) (12).

Bcr-Abl and c-Cbl coimmunoprecipitated with anti-CrkL antibodies but not with anti-c-Crk-II antibodies. This may indicate a specific role for CrkL, the product of a homologous but distinct gene (49), in chronic myelogenous leukemia, in the formation of a complex containing c-Cbl, Crk, and Bcr-Abl. Since both c-Crk and CrkL can be constitutively associated with the nucleotide exchange factors C3G and Sos through their SH3 domains in K562 cells (9, 48), a physiological role for the c-Cbl-Crk complex in Bcr-Abl tyrosine phosphorylation-mediated transformation may involve targeting the activation of $p21^{ras}$ or a member of the *ras* family of GTPases.

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