Interleukin-2 Stimulation Induces Tyrosine Phosphorylation of p120-Cbl and CrkL and Formation of Multimolecular Signaling Complexes in T Lymphocytes and Natural Killer Cells*

(Received for publication, June 5, 1997, and in revised form, October 31, 1997)

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Interleukin (IL)-2, a major growth and differentiation factor for T lymphocytes, was found to induce tyrosine phosphorylation of the proto-oncogene products p120-Cbl and CrkL in IL-2-dependent cell lines. We established that, in unstimulated lymphocytes, the Src homology 2 (SH2) and SH3 domain-containing protein Grb2 and the p85 subunit of phosphatidylinositol 3-kinase, associate constitutively with Cbl via their SH3 domains. Furthermore, IL-2 stimulation increased the level of interaction of phosphorylated Cbl with the p85 SH2 domains, and we provide evidence that the preformed Cbl-Grb2 complex recruits the phosphorylated p52 Shc adaptor protein. In addition, we demonstrate that the SH2-SH3-SH3 adaptor protein CrkL is tyrosine-phosphorylated in an IL-2-dependent manner and, via its SH2 domain, associates with a large proportion of phosphorylated Cbl. We also show that p85 is preassociated with the CrkL SH3 domain. Furthermore, the association of CrkL and p85 is increased after IL-2 treatment by a mechanism involving intermediary tyrosine-phosphorylated proteins that remain to be identified. Our results show that CrkL associates independently with Cbl or p85 and suggest that it also participates in larger complexes containing Cbl and p85.

Although the precise roles of Cbl and CrkL remain to be elucidated, their tyrosine phosphorylation, in addition to the multiple protein interactions described here, strongly suggest that Cbl and CrkL may play pivotal roles in the early steps of IL-2 signal transduction.

Interleukin (IL)1-2 is a major cytokine primarily involved in regulating T lymphocyte proliferation and differentiation. Its biological effects are mediated through a high affinity receptor, which comprises three polypeptide chains: α, β, and γ (1, 2). IL-2 signal transduction is initiated by the β- and γ-chains and has been widely studied in the past few years. The first steps of IL-2 signaling depend upon activation of the JAK and Src family kinases, which presumably phosphorylate the receptor itself and lead to activation of the Stat and Ras-mitogen-activated protein kinase pathways, respectively (2–7). In addition, IL-2 increases the lipid-kinase activity of phosphatidylinositol 3-kinase (PI3K). This increased activity is best detectable in anti-phosphotyrosine antibody immunoprecipitates, indicating either that the p85 subunit of PI3K becomes phosphorylated on tyrosine residues or that it binds to phosphoproteins in response to IL-2 (8–10). During the course of studies aimed at further defining IL-2 signal transduction, we observed IL-2-inducible phosphorylation of an approximately 120-kDa protein that appeared as a major tyrosine kinase substrate in T lymphocytes. Since the p120-Cbl oncogene product has previously been reported to be phosphorylated in response to various stimuli and can associate with p85-PI3K, we investigated whether Cbl might be involved in IL-2 signaling.

c-Cbl was first identified as the cellular homolog of the murine Csa NS-1 retrovirus oncogene (11). Its protein product, p120-Cbl contains several domains of interest, including a nuclear localization signal, a zinc finger, and a leucine zipper, suggesting that Cbl might function as a transcription factor, although no direct evidence for such an activity has yet been reported (12). In addition, Cbl contains a proline-rich motif and several potentially phosphorylatable tyrosine residues that could mediate protein-protein interactions (13). Indeed the proline-rich motif has been shown to be recognized by various SH3 domains, in particular those present in the adaptor proteins Grb2 and Nck, Src, and p85-PI3K (14–17). When phosphorylated, Cbl also associates through phosphotyrosine/SH2 interactions with Fyn, p85-PI3K, and Crk (17–20). Crk has recently been identified as a major Cbl-interacting protein and represents a family of SH3 and SH2 domain-containing adaptor proteins. Members of this family include two different splice products from the c-crk gene, Crk-I and Crk-II, as well as the related protein CrkL (21–23).

Although the function of p120-Cbl in signal transduction remains to be elucidated, p120-Cbl has clearly been shown to become phosphorylated on tyrosine residues in BCR-ABL transformed cells (24) and in several cell types upon stimulation with a variety of growth factors. This is the case for colony-stimulating factor-1, granulocyte-macrophage colony-stimulating factor, erythropoietin, or IL-3 stimulation in hemopoietic cells (25–27) and epidermal growth factor and insulin stimulation of other cell types (28–31). Furthermore, triggering of a number of cell membrane receptors in hemopoietic cells also leads to Cbl phosphorylation. These include the antigen receptors on B (membrane Ig) and T (TCR-CD3) lymphocytes (32, 33) and CD38 or Fcy receptors on monocyte-derived cell lines (34, 35).

In this report we have used the human IL-2-dependent Kit
225 T lymphocyte and NKL natural killer cell lines to investigate the regulation of Cbl by interleukin-2. We report here that p120-Cbl is rapidly tyrosine-phosphorylated in response to IL-2 stimulation in these cells. In addition, Cbl is constitutively associated with Grb2, and IL-2 stimulation induces the recruitment of tyrosine-phosphorylated Shc to the Cbl-Grb2 complex. Finally, IL-2 stimulation appears to regulate the ability of Cbl to associate with p85-P13K in vitro and in vivo. IL-2 also induces the phosphorylation of CrkL and regulates its association with p85 and Cbl through different molecular interactions. Thus, our study identifies Cbl and CrkL as important tyrosine kinase substrates in IL-2-responsive cells and suggests that Cbl and CrkL, which associate with multiple partners, may function as essential docking proteins in IL-2 signal transduction.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—The human T cell chronic lymphocytic leukemia-derived, IL-2-dependent Kit 225 cell line was kindly provided by Dr. T. Hori (Kyoto University, Japan; Ref. 36). The NKL cell line, established from the peripheral blood of a patient with large granular lymphocyte leukemia, was kindly provided by Dr. J. Ritz (Dana-Farber Cancer Institute, Boston, MA; Ref. 37). Kit 225 and NKL cells were maintained in culture medium (RPMI 1640, 10% fetal calf serum and antibiotics) supplemented with 0.5 nM recombinant human IL-2 (generously provided by P. Ferrara, Sanofi, France). Cells were seeded in culture medium at 2 \times 10^7/ml in cold 0.5% Triton lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 0.5% Triton X-100, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM vanadate, and 5 μg/ml each leupeptin, aprotinin, and pepstatin). Lysis was performed for 30 min by washing three times and resuspending the cells in culture medium without IL-2 at 2 × 10^5/ml for 48 h. The Jurkat T cell line was grown in culture medium.

Cell Stimulation—IL-2-deprived Kit 225 or NKL cells were suspended in culture medium at 2 × 10^7/ml and incubated at 37 °C without (control) or with 1 nM recombinant IL-2 for various periods of time as indicated. Cells were harvested by centrifugation and lysed at 5 × 10^7/ml in cold 0.5% Triton lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 0.5% Triton X-100, 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM vanadate, and 5 μg/ml each leupeptin, aprotinin, and pepstatin). Lysis was performed for 30 min on ice. Lysates were clarified by centrifugation at 15,000 × g for 20 min at 4 °C. Jurkat cells, resuspended at 2 × 10^7/ml, were stimulated for the indicated times by incubation with UCHT-1 antibody at 10 μg/ml and lysed as described above.

Reagents and Antibodies—Polyclonal antibodies against Cbl (Sc-170), Grb2 (Sc-422, used for immunoprecipitation), and Crkl (Sc-319) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies to Grb2 (G 16720, used in Western blot) and Crk-L (Sc-170) antibody was from Immunotech (Marseille, France). The polyclonal antibody 4G10 showed a prominent tyrosine-phosphorylated protein. The anti-phoshotyrosine antibody was obtained from Upstate Biotechnology Inc. (UBI, Lake Placid, NY), and the anti-Cbl UCHT-1 antibody was from Immunotech (Marseille, France). The anti-phosphotyrosine antibody 4G10 was initially provided by Dr. B. Druker (Oregon Health Sciences University, Portland, OR), and additional supplies were purchased from UBI.

The tyrosine 317 phosphopeptide (P59YVNVDP) derived from the human Shc protein, and the proline-rich peptide (VPFPVPPPRR) derived from the human SOS protein were synthesized as described previously (38, 39).

Immunoprecipitation and Immunoblotting—Cell lysates (5 × 10^7 cells equivalents) were preclarified with protein G-Sepharose. 1–10 μg of antibodies were added to the lysates. After 2 h of rocking at 4 °C, 20 μl of protein G-Sepharose was added, and incubation continued for an additional 45 min. Beads were washed five times with cold lysis buffer, and the precipitates were boiled in 2 × Laemml sample buffer, resolved by SDS-PAGE, and electrotransferred to nitrocellulose membranes (Amersham Corp.). Immunodepletion experiments were performed by three successive rounds of immunoprecipitation using protein G-Sepharose-adsorbed antibodies, followed by one round with beads alone, and the final immunoprecipitation was performed on the supernatant as described above. For immunoblotting, the membranes were blocked for 2 h at room temperature in either 5% nonfat dry milk or 3% bovine serum albumin in Tris-buffered saline (TBS), 0.5% Tween (TBS-T). Filters were washed four times in TBS-T and incubated for 1.5 h with optimal concentrations of primary antibodies diluted in TBS, 0.1% Tween. Following four additional washes in TBS-T, the filters were further incubated for 45 min with horseradish peroxidase-conjugated secondary antibodies (sheep anti-mouse Ig from Amersham or goat anti-rabbit Ig from DAKO). Visualization was performed using Amersham ECL reagents and autoradiographic films.

GST Fusion Proteins—A plasmid encoding for GST-Grb2 full-length fusion protein was kindly provided by Dr. P.-O. Couraud (Institut Cochin de Genétique Moléculaire, Paris, France). GST-CrkL constructs (CrkL (full-length), SH2, and N-terminal) were a kind gift from B. J. Druker (40). Construction of GST-phos was described in detail elsewhere. Briefly, the cDNA for human p85α and its various subdomains was amplified by PCR, and appropriate fragments were cloned into PGEX vectors (Pharmacia Biotech Inc.) to yield fusion proteins containing the full-length p85 (GST-phos), the SH3 domain (GST-SH3; amino acid residues 1–83), the BCR homology domain framed by the two proline-rich regions (GST-PBP; residues 73–336), and the two-SH2-containing C-terminal half of p85 (GST-SH2(N+C); residues 313–724). GST fusion proteins were produced and purified as described previously (41).

GST Pull-down Experiments—5–10 μg of purified GST proteins were adsorbed onto glutathione-Sepharose beads (Pharmacia). Precleared lysates were rocked with coupled beads for 2 h at 4 °C. The beads were washed 5 times with lysis buffer and boiled in 2 × sample buffer, and bound proteins were analyzed by Western blotting as described above.

RESULTS

Tyrosine Phosphorylation of Cbl in IL-2-stimulated Kit 225 Cells—Western blot analysis of total cell lysates from IL-2-stimulated Kit 225 cells with the anti-phosphotyrosine antibody 4G10 showed a prominent tyrosine-phosphorylated protein of approximately 120 kDa (not shown). The possibility that this 120-kDa signal contained Cbl was directly investigated by immunoprecipitation with anti-Cbl or 4G10 (anti-phosphotyrosine) antibodies and Western blotting with the reciprocal antibody. Fig. 1A shows that indeed Cbl was phosphorylated on tyrosine residues in a stimulation-dependent manner and could be recovered in 4G10 immunoprecipitates of IL-2-stimulated cell lysates. The kinetics of Cbl phosphorylation was then...
studied, and the results shown in Fig. 1B indicated that phosphorylation was detected as soon as 30 s, peaking around 10 min after IL-2 stimulation and slightly declining thereafter but still present for up to 60 min. To estimate the amount of Cbl that became phosphorylated in response to IL-2, two successive anti-phosphotyrosine immunoprecipitates were performed with lysates obtained from $2 \times 10^7$ cells, pooled, and run in parallel with total cell lysates from varying numbers of cells, and the Western blot developed with anti-Cbl antiserum was analyzed by densitometry scanning (data not shown). This series of experiments showed that approximately 2% of total Cbl could be immunoprecipitated by anti-phosphotyrosine antibodies following IL-2 stimulation.

Cbl also became tyrosine-phosphorylated in NKL cells, another IL-2-dependent human cell line derived from natural killer lymphocytes (Fig. 1B). Similar data were obtained in human phytohemagglutinin T cell blasts and murine CTLL-2 cells (not shown), demonstrating that Cbl phosphorylation is a general event in IL-2 signaling and excluding the possibility that these observations might result from unique and unidentified features of Kit 225 cells.

**Constitutive Association of Cbl with Grb2 and Stimulation-dependent Recruitment of Phosphorylated p52Shc**—To investigate whether Cbl associates with other phosphorylated proteins in response to IL-2, Cbl immunoprecipitations were performed under conditions slightly modified from those shown in Fig. 1. Cell lysates from $5 \times 10^7$ Kit 225 cells corresponding to 5 times more proteins were used for each immunoprecipitation point. Under these conditions, anti-phosphotyrosine immunoblotting revealed two additional bands that specifically co-precipitated with Cbl (Fig. 2A). One band migrating with an apparent molecular mass of 70–75 kDa appears associated with Cbl regardless of IL-2 stimulation and has so far not been identified. The other band migrated around 50–52 kDa at the same position as a signal present in anti-Grb2 immunoprecipitates (open arrowheads), suggesting that it might be the adaptor protein Shc. Indeed Shc associates with the Grb2 SH2 domain and is known to be phosphorylated in response to IL-2 (42, 43). Lymphocytes do not express the p66 isoform of Shc, and p52 Shc migrates very close to the immunoglobulin heavy chain signal (position indicated by Ig-H on all figures), making it difficult to identify by direct blotting of immunoprecipitates. To formally identify this band as Shc, immune complexes obtained with anti-Cbl antibodies were dissociated by boiling in 2% SDS, and the eluted proteins were diluted 20-fold in lysis buffer and reprobed with anti-Shc antibodies. As seen in Fig. 2B, the 52-kDa phosphoprotein present in Cbl immunoprecipitate was indeed recognized by the anti-Shc antibody in the second immunoprecipitation and migrated exactly at the same level as phosphorylated p52Shc in the positive control experiment.

The IL-2-induced interaction of Shc with Cbl led us to investigate whether Cbl might be associated with Grb2 in Kit 225 cells. Initial experiments indeed showed that Cbl immunoprecipitates contained Grb2, and Grb2 immunoprecipitates contained Cbl, although the association appeared to occur with a low stoichiometry. To better analyze this interaction, Cbl and Grb2 immunoprecipitates were split in two fractions of $\frac{1}{10}$ and $\frac{3}{10}$ of the material, which were run in parallel gels. The membrane containing $\frac{1}{10}$ of the Cbl immunoprecipitates and $\frac{3}{10}$ of the Grb2 immunoprecipitates was blotted with Cbl antibodies (Fig. 2C, top), and the membrane containing $\frac{3}{10}$ of the Grb2 immunoprecipitates and $\frac{1}{10}$ of the Cbl immunoprecipitates was blotted with Grb2 antibodies (Fig. 2C, bottom). This approach, combined with densitometry scanning indicated that approximately 3% of total Grb2 was associated with Cbl.

To further understand how Cbl interacts with Grb2 and Shc, GST-Grb2 fusion proteins were incubated with cell lysates from resting or IL-2-stimulated cells, and the nature of binding proteins was assessed by Western blot. The pattern of tyrosine-phosphorylated proteins that bound to GST-Grb2 was quite complex (Fig. 2D) and very similar to that seen in Grb2 immunoprecipitates (Fig. 2A). Of particular interest, a 120-kDa signal appears, depending upon IL-2 stimulation, which represents phosphorylated Cbl. Reproducing the same membrane with anti-Cbl antibodies demonstrated that Cbl actually associates with GST-Grb2 regardless of IL-2 stimulation. Thus, as suggested by the immunoprecipitation experiments described above, Grb2/ Cbl interaction is independent of Cbl phosphorylation.

To analyze the respective involvement of Grb2 SH3 and SH2 domains in the observed protein interactions, we took advantage of the ability of defined peptide sequences to selectively block each of these domains. A proline-rich peptide derived from the SOS protein was used to block Grb2 SH3 domains, and a phosphorylated tyrosine 317-containing peptide derived from Shc was used to block Grb2 SH2 as described under “Materials and Methods.” Control experiments performed with GST-Grb2 indicated that the proline-rich peptide, when added at a concentration of 100 μM, clearly prevented the binding of Cbl to GST-Grb2, whereas the Shc-derived phosphorylated peptide reduced the binding of Shc but had no effect on Cbl.
binding to Grb2 (not shown). To understand the nature of Cbl/Grb2/Shc interactions in vivo, we investigated the effects of these two peptides on the formation of this complex in resting and IL-2-stimulated cells. To this end, appropriate concentrations of each of these two peptides were added to cell lysates, and Cbl was immunoprecipitated (Fig. 3). In the presence of the proline-rich peptide, Grb2 became undetectable in the Cbl immunoprecipitate, indicating that the Cbl/Grb2 interaction was dramatically displaced by the SOS peptide (lanes 5–6). Under these conditions, however, all of the Grb2 may not be prevented from binding as suggested by the presence of a residual amount of phosphorylated Shc. Adding the phosphorylated Shc peptide also yielded very clear cut results. This peptide did not affect the Cbl/Grb2 interaction at all yet completely removed Shc from the immunoprecipitated complex (lanes 7 and 8). Adding both peptides together induced a complete dissociation of Grb2 and Shc from Cbl. Taken together, the results of this series of experiments demonstrated that the Cbl/Grb2 interaction is probably mediated through recognition of Cbl proline-rich sequences by Grb2 SH3 domains and that IL-2-dependent phosphorylation of Shc induces its association, through the Grb2 SH2 domain, to the preformed Cbl-Grb2 complex.

These experiments also showed that the unidentified pp70–75 probably does not interact directly with Cbl but might be bound to the complex through interaction with the Grb2 SH2. Indeed, it was completely absent from the Cbl immunoprecipitate whether Grb2 was displaced by the SOS peptide or in the presence of the phosphorylated Shc peptide (Fig. 3).

Association of Cbl with p85 in IL-2 or TCR-stimulated T Cell Lines—Since Cbl has been shown to associate with p85-P13K in response to various stimuli, we investigated whether this was also the case in IL-2-stimulated Kit 225. The modification of Cbl/p85 interaction induced by IL-2 in Kit 225 cells was somewhat difficult to study, since no Cbl could be detected in anti-p85 immunoprecipitates, whether cells were stimulated or not. However, the converse experiment indicated that a small proportion of p85 could be precipitated by Cbl antibodies. Although not seen on the exposure shown in Fig. 4A, but confirmed in Fig. 5, the Cbl/p85 association was detected at a low level in resting cells. Furthermore, IL-2 induced a clear increase of the amount of p85 present in the Cbl immunoprecipitate (Fig. 4A, lanes 11 and 12). Because of the relative weakness of these signals, we sought to compare the effects of IL-2 stimulation to those induced by triggering the TCR-C3 complex in Jurkat cells, which have been well described (44). The effects of CD3 stimulation could not be assessed directly with Kit 225 cells, which do not respond to stimulation by the UCHT-1 antibody, and these experiments were therefore performed in Jurkat cells. In these cells, Cbl is constitutively phosphorylated to a fairly high level (data not shown) and associated with p85 regardless of stimulation as seen in Fig. 4A, lanes 3 and 5. Nevertheless, stimulation of Jurkat cells with the anti-CD3 UCHT-1 antibody resulted in a detectable increase in Cbl phosphorylation (not shown) and in an increased association with p85. This was best evidenced by Cbl immunoblotting of anti-p85 immunoprecipitates (Fig. 4A, lanes 3 and 4, top), and some increase in p85 was also seen in Cbl immunoprecipitates (lanes 5 and 6). Thus, we have shown that in Kit 225 cells, IL-2 regulates Cbl/p85 interaction, albeit to a significantly smaller extent than TCR stimulation does in Jurkat cells.

To further analyze how Cbl interacts with p85-P13K, we used several GST fusion proteins consisting of the individual subdomains of p85, as described under "Materials and Methods." Fusion proteins were incubated with lysates from resting or IL-2-stimulated Kit 225 cells, and Cbl was revealed by Western blotting as above. As shown in Fig. 4B (bottom), GST-p85, as well as GST-SH3, recognized Cbl in lysates from nonstimulated cells, whereas control GST, GST-PBP, or GST-SH2(N + C) did not pull down Cbl. Furthermore, the amount of Cbl binding to GST-p85 increased in IL-2-stimulated cell lysates and GST-SH2(N + C) bound Cbl only when it was phosphorylated, i.e. in IL-2-stimulated cells. The level of interaction of Cbl with GST-SH3 appeared identical whether cells had been stimulated or...
not. It should be noted that when probed with anti-phosphotyrosine antibody, Cbl was the main phosphoprotein bound to GST-SH3 (Fig. 4 lane 4) as opposed to the complex pattern of proteins recognized by GST-p85 and GST-SH2(N + C). Thus, the basal association of Cbl with p85 may involve the Cbl proline-rich motif and the p85 SH3 domain, whereas the increase of the interaction induced by IL-2 depends on p85 SH2 domains that may recognize phosphorylated tyrosine residues in Cbl.

Regulation of Cbl Interaction with CrkL by IL-2—We then investigated the association of Cbl with proteins of the Crk family using anti-Crk-I/II or anti-Crkl antibodies. As shown in Fig. 5, a small amount of Cbl appeared associated with all Crk proteins in resting Kit 225 cells. Following IL-2 stimulation, the amount of Cbl immunoprecipitated by anti-Crkl-I/II was only slightly increased, whereas a dramatic augmentation of Cbl was observed in CrkL-specific immunoprecipitates (Fig. 5, anti-Cbl and 4G10 immunoblots). In additional experiments (not shown), it was determined that approximately 1% of total Cbl became associated with CrkL in response to IL-2 and that most of it was indeed phosphorylated Cbl (compare 4G10 signals in lanes 4 and 6 in Fig. 5). As previously reported by others (30), and possibly due to the stoichiometry of the complexes, the reciprocal experiment failed to identify either Crk or CrkL in Cbl immunoprecipitates. Furthermore, we could evidence a constitutive association of p85 with Crk and Crkl (IB, p85 panel), and this interaction was noticeably increased with CrkL after IL-2 treatment. In these experiments, the use of a monoclonal antibody to immunoprecipitate Crk-I/II prevented identification of Grb2, which was masked by the Ig light chain signal. In CrkL immunoprecipitates, however, Grb2 was detected in resting cells and increased after IL-2 stimulation, probably as a result of the larger amount of Cbl coprecipitated with CrkL under these conditions.

Immunoblotting with anti-phosphotyrosine provided more information about Crk proteins. First, CrkL, but not Crk-I/II, appeared to be tyrosine-phosphorylated in response to IL-2. CrkL migrated as a doublet at 39 and 42 kDa, and the phosphorylated form appeared as an additional band of intermediate molecular mass in the CrkL immunoblot (shown by the black arrowhead in Fig. 5, lanes 2 and 4). CrkL was present in anti-Crk-I/II immunoprecipitates due to cross-reactivity of the anti-Crk-I/II antibody.

Furthermore, besides Cbl and Shc, anti-CrkL coprecipitated several phosphorylated proteins with apparent mass of 100, 85, and 68 kDa. Association with these phosphoproteins, which remain to be identified, suggests that CrkL might serve additional adaptor functions in IL-2 signaling.

CrkL Interaction with Cbl and p85—The data reported above indicate that Cbl interacts with p85 and that both Cbl and p85 could be co-precipitated with CrkL. It was therefore of interest to try to understand how these proteins associated with each other and particularly which of these interactions were direct or mediated by one of the partners. The first indication came from kinetics analysis displayed in Fig. 6, showing the time dependence of CrkL phosphorylation and association of p85 and Cbl. In nonstimulated cells (time 0), p85 was detectable in CrkL immunoprecipitates in the absence of Cbl, demonstrating a CrkL/p85 association, which is not mediated by Cbl, independent of tyrosine phosphorylation, and is likely to occur through CrkL SH3 recognition of p85 proline-rich sequences as shown below and in Ref. 45. Following IL-2 stimulation, the amount of p85 associated with CrkL increased rapidly. In contrast, association of Cbl increased progressively to reach a maximum at 10 min after stimulation, slightly declining thereafter, which correlates with the kinetics of Cbl phosphorylation, as shown in Fig. 1.

Experiments were then undertaken where cell lysates were first depleted either of Cbl or of p85 by three rounds of immunodepletion procedure with the appropriate antibodies prior to immunoprecipitation of CrkL from the depleted supernatants. Efficiency of this immunodepletion procedure was assessed by Western blotting of depleted cell lysates, as well as by control immunoprecipitations with the relevant antibodies (data not shown). p85 was clearly identified in CrkL immunoprecipitates from Cbl-depleted lysates (Fig. 7A, lanes 7 and 8), and reciprocally, Cbl was detected in CrkL immunoprecipitates from lysates that had been depleted of p85 (Fig. 7A, lanes 5 and 6). These results indicate that CrkL associates with p85 or Cbl independently of each other. However, they do not provide any
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FIG. 7. CrkL simultaneously and independently associates with Cbl and p85. Kit 225 cells were either left unstimulated (−) or were incubated (+) for 5 min with IL-2 A and B, lysates from 2 × 10^7 (A) or 5 × 10^6 (B) cells were immunodepleted (Pre IP) with the indicated antibodies. The recovered supernatants were immunoprecipitated (IP) with the indicated antibodies, separated, and transferred to nitrocellulose. Whole cell lysates (WCL) corresponding to 5 × 10^6 cells were run in parallel. The filters were cut into size region pieces and probed as indicated. C, lysates from 5 × 10^6 cells were incubated with GST fusion proteins immobilized on glutathione-Sepharose beads as described, separated, transferred, and probed with either anti-Cbl or anti-p85 antibodies.

information as to whether a ternary Cbl-p85-CrkL complex may exist, in which CrkL could mediate Cbl/p85 association, or whether p85 and Cbl interact directly, or at least independently of CrkL. To investigate this possibility, a similar approach was attempted, where cell lysates were depleted of CrkL before immunoprecipitation with p85 or Cbl antibodies. These experiments were rendered somewhat difficult due to the low stoichiometry of the p85/Cbl interaction, which required that large quantities of cell lysate be used. Under these conditions, and given the large amount of CrkL present in Kit 225 cells, depletion of CrkL could only be achieved to an estimated 80–90% completion (Fig. 7B, lanes 11–14). The data obtained in this series of experiments indicated that CrkL depletion does not affect the Cbl/p85 association that is seen in nonstimulated cell lysate, since p85 was detected in Cbl immunoprecipitate to a similar level, before (lane 7) and after (lane 9) CrkL depletion. In contrast, the higher level of Cbl/p85 association seen in lysates from IL-2-stimulated cells is clearly reduced following CrkL depletion (lanes 8 and 10). A tentative interpretation of these results would be that Cbl interacts directly with p85 under basal conditions, whereas IL-2 stimulation induces an indirect association involving CrkL.

The nature of the CrkL/p85 and CrkL/Cbl interactions was then investigated using GST-CrkL constructs in pull-down experiments. In Fig. 7C, we demonstrate that Cbl associates with the CrkL SH2 domain in an IL-2-dependent manner, whereas no Cbl could be detected on the SH3 domain. On the other hand (Fig. 7C, bottom), we show that the CrkL-p85 complex is mainly due to the SH3 domain of CrkL, which is probably interacting with p85 proline-rich motifs. Thus, these results indicate that CrkL interacts with phosphorylated Cbl via its SH2 domain and is constitutively associated with p85 through its SH3 domain. In these in vitro experiments, however, no increased binding of p85 could be demonstrated in response to IL-2, suggesting that increased binding seen in immunoprecipitations described above may be indirect and poorly detectable under these conditions.

DISCUSSION

In this report, we established that IL-2 stimulation of T lymphocytes and of natural killer cells induces tyrosine phosphorylation of p120-Cbl. Whereas similar findings have been reported in a number of cell types stimulated by various growth factors, only triggering of the CD3-TCR complex has, to our knowledge, been shown to regulate Cbl in T cells. In our study, Cbl phosphorylation was best evidenced in two human cell lines, Kit 225 and NKL, possibly because these cells can withstand IL-2 starvation for as long as 48 h without undergoing apoptosis. Under IL-2-free culture conditions, cells from both cell lines accumulate in the G1 phase of the cell cycle and therefore acquire the characteristics of true resting cells (37). In a number of other IL-2-responsive cells, Cbl was more difficult to study, since it remained tyrosine-phosphorylated after IL-2 deprivation up to a point where the cells had entered apoptosis and could not be restimulated.

Cbl has recently been shown to associate with and to be phosphorylated by, Src or Syk family kinases (16, 45, 46). Although the identity of the kinase(s) responsible for Cbl phosphorylation in our study has not been addressed, it is possible that it belongs to one of these two important kinase families that are known to be activated by IL-2 (2, 47). Recent reports indicate that Cbl is phosphorylated under various conditions in several cell types, suggesting that Cbl plays an important role in receptor-mediated signal transduction, although its precise function has not yet been established. Blake et al. (12) have reported that a truncated form of Cbl localized to the nucleus, an observation that, taken together with the structural features of Cbl, would be consistent with its functioning as a transcription factor. We have obtained no evidence for Cbl nuclear translocation in IL-2-stimulated cells, and this hypothesis has not been further substantiated. Most of the currently available information would point to a role for Cbl in early signaling events. As shown here, Cbl exists in complexes with Grb2 and Crk. Grb2, Crk, and CrkL are adaptor proteins known to bind important regulators of p21^ras and Ras-related small G proteins, such as the exchange factors SOS, Vav, and C3G (48), respectively, and it has been speculated that Cbl might participate in negative feedback regulation of the Ras pathway (49). However, Ueno et al. (50) recently reported that modulating Cbl expression had no effect on Ras but regulated the JAK-Stat pathway in epidermal growth factor-stimulated NIH3T3 cells. Other studies similarly point to a regulatory role of Cbl upon the platelet-derived growth factor receptor or other nonreceptor tyrosine kinases such as Syk (51, 52). A better understanding of the role of Cbl in normal cells may come from the identification of possible partners for Cbl in signaling complexes that associate following receptor stimulation.

We have observed that in resting Kit 225 cells, Cbl is constitutively associated with Grb2 through interaction of the Grb2-
SH3 domain presumably with a class II-proline-rich motif in Cbl (PPVPPR; residues 494–499). IL-2 stimulation does not modify this interaction, but it induces the association of tyrosine-phosphorylated Shc to the Cbl-Grb2 complex. Using competitive synthetic peptides, we demonstrated that this association was dependent upon recognition of Shc-phosphorylated tyrosine 317 by the Grb2 SH2 domain. The nature of both Cbl/Grb2 and Grb2/Shc interactions was established not only in vitro using GST fusion proteins but was also shown to occur in vivo as demonstrated in Cbl immunoprecipitates. In the Cbl-Grb2-Shc complex, both the SH2 domain of Shc and its phosphorytost binds other phosphorylated partners. One of these putative partners might be the IL-2 receptor itself, which contains an NQGY Shc-phosphorytose binding domain target sequence at tyrosine 338 of its β-chain (53). Although no evidence has been obtained that Cbl interacts with the IL-2 receptor, the possibility exists that Cbl may be one of the numerous proteins recruited by this receptor upon stimulation.

In addition, we have observed that Cbl binds to p85-Pi3K through two types of interactions. A constitutive association was found in resting cells, and pull-down experiments using GST-p85 subdomains indicated that it was mediated by the p85 SH3. Cell stimulation with IL-2 increased the binding of p85 to Cbl, as seen by immunoprecipitation experiments. We also found that phosphorylated Cbl bound to the full-length p85 fusion protein and to the p85 SH2(N + C) domain in vitro. This was not unexpected, since Cbl contains two YXXM sequences (tyrosine residues 371 and 731), which represent canonical p85 SH2 targets (54). It is noteworthy that although Cbl/p85 interactions have not been studied in great detail, differences are observed in various cell type/growth factor combinations. For instance, a similar level of constitutive association has been described in the murine hemopoietic precursor 32D cell line, but IL-3 stimulation, which also induces Cbl phosphorylation, does not appear to regulate its association with p85 (27). The precise role of Cbl/p85 interactions is currently unclear. It is however possible that, at least for IL-2 signaling, Cbl might be involved in recruiting Pi3K to the receptor. Indeed, Pi3K is activated in response to IL-2, despite the absence of YXXM motifs on either chain of the IL-2 receptor, and no candidate adaptor protein has been identified so far. Consistent with this hypothesis, we have observed that, although Cbl is mainly found as a cytosolic protein, phosphorylated Cbl and p85 are co-detected in cell membrane fractions after IL-2 stimulation (data not shown).

The SH3 and SH2 domain-containing Crk family of adaptor proteins have also been described as associating with Cbl in a stimulation-dependent manner. We report here that phosphorylated Cbl associates with CrkL following stimulation. This association is likely to be mediated by CrkL SH2 recognition of YXXP motifs present at residues 337, 700, and 774 in Cbl (11), which accounts for the observation reported above that a large proportion of Cbl found in association with CrkL is indeed phosphorylated. In addition, CrkL was found to interact constitutively as well as in an IL-2-dependent manner with the p85 subunit of phosphatidylinositol 3-kinase. IL-2 stimulation results in an increased association of CrkL with p85, which is unlikely to be mediated directly by the CrkL SH2 domain as seen in GST pull-down experiments. Cbl immunodepletion experiments also indicated that although Cbl and p85 do interact with each other, Cbl does not participate significantly in the observed CrkL/p85 association. CrkL does not contain consensus YXXM target sequences for p85 SH2 domains and is not pulled down by GST-p85 SH2(N + C), yet either 50 μM phenylphosphate or a 10 μM concentration of a phosphorylated YMDM-containing peptide was found to prevent the co-precipitation of p85 with CrkL antibodies (data not shown). It is thus clearly possible that the phosphotyrosine-dependent interaction between CrkL and p85 is mediated by another protein.

According to this hypothesis, CrkL was shown to associate with at least three additional phosphoproteins, the identification of which is currently in progress. Our data demonstrate that IL-2 regulates at least three types of complexes: CrkL-p85, CrkL-Cbl, and Cbl-p85. In addition, the Cbl-p85 complex may contain another adaptor protein, as a CrkL-associated protein, as shown in CrkL immunodepletion experiments. Thus, IL-2 regulates the formation of a variety of complexes, involving all of the possible interactions between the functional domains of these three molecules, in ways that are very similar to what has recently been described in BCR-ABL transformed cells (55).

Furthermore, we show here that IL-2 stimulation results in the specific phosphorylation of CrkL but not of Cbl. It is of interest to note that CD3-TCR stimulation of T lymphocytes has been reported not to induce the phosphorylation of Crk or CrkL, ruling out the possibility that this observation might be related to a cell type-specific effect (56). Rather, the phosphorylation of CrkL, which has already been reported in BCR-ABL transformed cells and in signaling by Steel factor and epidermal growth factor (30, 57, 58) represents, in T lymphocytes, a unique feature of IL-2 signaling. Further studies should directly address the role of CrkL phosphorylation as well as the possibility that CrkL might be involved in other IL-2-induced signaling pathways, since we have observed here that CrkL associates with at least three additional, as yet unidentified, tyrosine-phosphorytosed proteins. This also applies to Cbl, which interacts through Grb2 with a p70–75 phosphoprotein that preliminary experiments, using a series of antibodies to known proteins of a similar Mr, have so far failed to identify. Taken together, the data reported here indicate that through their regulated interactions with multiple signaling partners including Grb2, Shc, and the p85 subunit of phosphatidylinositol 3-kinase, Cbl and CrkL may have central functions in IL-2 signaling.

Acknowledgments—We thank T. Hori for kindly providing the Kit 225 cell line and J. Ritz and J. Breard for making NKL cells available for this study. We also thank B. Druker, P-O. Couraud, and P. Ferrara for essential reagents used in this study; Wanging Liu for peptide synthesis; and J. Pierre, M. Pallardy, T. Reid, and J-L. Zugaza for critical reading of the manuscript.

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J. Biol. Chem. 1998, 273:3986-3993.
doi: 10.1074/jbc.273.7.3986

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