Phosphorylation of Cbl following Stimulation with Interleukin-3 and Its Association with Grb2, Fyn, and Phosphatidylinositol 3-Kinase*

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We have demonstrated that a 120-kDa protein, identified as Cbl, becomes rapidly phosphorylated on tyrosine residues following stimulation of factor-dependent cells with interleukin-3 (IL-3). Little or no phosphorylation of Cbl was observed in the absence of IL-3 stimulation and phosphorylation is maximal by 20–30 min after IL-3 stimulation. Association of Cbl with Grb2 was noted in unstimulated cells, and the amount of Cbl associated with Grb2 increased following IL-3 stimulation. The p85 subunit of phosphatidylinositol 3-kinase was constitutively associated with Cbl. Approximately 10% of the PI kinase activity present in anti-phosphotyrosine immunoprecipitates was present in anti-Cbl immunoprecipitates of IL-3-stimulated cells. The constitutive association of Cbl with Fyn was also observed. Cbl was observed to bind to bacterial fusion proteins encoding the unique, SH3, and SH2 domains of Fyn, Hck, and Lyn. The SH2 domain of Fyn alone was able to bind Cbl to nearly the same extent as did the fusion protein encoding the unique, SH3, and SH2 domains of Lyn. This was not the case for the SH2 domain of Hck, however, as binding of the Hck fusion protein to Cbl appeared to require multiple domains. The binding of the fusion proteins to Cbl occurred regardless of whether Cbl was tyrosine-phosphorylated or not, and the binding could not be disrupted by the addition of 30 μM free phosphotyrosine. These data suggest the unexpected conclusion that the Fyn SH2 domain may bind to Cbl in a phosphotyrosine-independent manner.

Stimulation of type 1 cytokine family receptors with their ligands results in the rapid tyrosine phosphorylation of multiple cellular proteins including the receptors themselves. Since these receptors do not encode intrinsic protein-tyrosine kinase activity (1–8), the activation of nonreceptor tyrosine kinases, such as the Janus and Src families of tyrosine kinases, is critical in signaling events. Investigations utilizing cell lines lacking specific Janus family members has indicated that they are critical in signal transduction response to cytokines (9, 10). Similar studies have not been conducted to date with Src-like kinases. In addition to understanding which tyrosine kinases are activated, the identification of downstream signaling molecules is fundamental to understanding signal transduction. We have been investigating the activation of Src-like kinases in signal transduction by the IL-3 receptor. Following IL-3 stimulation of the murine myeloid cell line 32D c3, we have observed the activation of three Src-like kinases: Fyn, Hck, and Lyn (11). As part of a study to examine the interaction of Src-like kinases with the β subunit of the IL-3 receptor, we observed that a tyrosine-phosphorylated protein of 120 kDa bound to bacterial fusion proteins containing the unique, SH3, and SH2 domains of Fyn, Hck, and Lyn. The current study was initiated to determine whether this protein was Cbl.

The Cbl protein represents the cellular homologue of the oncogene present in the Cas-NS-1 retrovirus (12). Sequence analysis of the Cbl cDNA revealed that the protein contains 913 amino acids, a putative nuclear localization sequence in its N-terminal region, a “RING finger” motif typical of numerous DNA-binding proteins, and several proline-rich sequences in its C-terminal half that may serve as SH3 domain binding sites (12). In spite of the presence of a nuclear localization signal and a DNA-binding motif, there is no evidence that Cbl is present in the nucleus or that it binds to DNA (12, 13). Recent studies have demonstrated that Cbl becomes tyrosine-phosphorylated following stimulation of the following receptors: the T-cell receptor (14–16), the B-cell receptor (17, 18), the Fc receptor (19, 20), the epidermal growth factor receptor (21–24), the erythropoietin receptor (25), and the receptor for granulocyte-macrophage colony-stimulating factor (25). The Cbl protein is also phosphorylated in cells expressing either v-Abl or BCR-ABL (26, 27). In receptor-stimulated cells, Cbl has been observed to associate with a variety of proteins by either co-immunoprecipitation studies or binding to bacterial fusion proteins. Association has been observed with PI 3-kinase in a phosphotyrosine-dependent manner via SH2 and SH3 domains (14, 15, 21, 24, 27), with the SH3 domain of Lyn (19, 20), with the SH2 and SH3 domains of Fyn (15, 19), with the SH2 domains of Crk (27), and with Grb2 via one of its SH3 domains (16, 18, 24, 25). The association of Cbl with the p85 subunit of PI 3-kinase and Grb2 suggests that phosphorylation of Cbl may regulate activation of PI 3-kinase and Ras. It is not clear whether Cbl can associate with all of these molecules in the same cell, or in different cell types. In this report, we demonstrate that Cbl is the 120-kDa phosphoprotein observed in IL-3-stimulated cells. Cbl was observed to associate with Grb2, Fyn, and PI 3-kinase. The association of Cbl with Grb2 following cytokine stimulation is consistent with a previous report (25); however, the described

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†The abbreviations used are: IL-3, interleukin-3; GST, glutathione S-transferase; GM-CSF, granulocyte-macrophage colony-stimulating factor; PI, phosphatidylinositol; PI 3-kinase, phosphatidylinositol 3'-kinase; SH2, Src homology 2; SH3, Src homology 3; PIPES, 1,4-piperazinediethanesulfonic acid; RIPA, radioimmune precipitation buffer.

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interaction Cbl with Fyn and PI 3-kinase following cytokine stimulation is novel to this report. The association of Cbl with Fyn may be mediated by the SH2 domain of Fyn binding to Cbl in a phosphotyrosine-independent manner.

**MATERIALS AND METHODS**

**Cells and Cell Culture—**The 32D c3 e13 cell line was obtained from Dr. Joel Greenberger (University of Pittsburgh, Pittsburgh, PA) and was maintained as described (28). Recombinant murine IL-3 was obtained from Collaborative Biomedical Products (Bedford, MA).

**Immunoprecipitation and Immunoblotting—**Immunoprecipitation was performed as described previously (11). Cells were lysed either with RIPA (150 mM NaCl, 50 mM Tris (pH 7.4), 2 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate) or with EB (50 mM NaCl, 10 mM Tris (pH 7.4), 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate). Both lysis buffers were supplemented with 100 units/ml aprotinin (Calbiochem, La Jolla, CA). Rabbit anti-Lyn, rabbit anti-p56 lck of PI-3 kinase, rabbit anti-Grb2, rabbit anti-Fyn coupled to agarose beads, and anti-phosphotyrosine monoclonal antibody 4G10 coupled to agarose beads were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies to Cbl and Hck were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Additional antibodies to PI 3-kinase, Lyn, and Grb2 were obtained from Transduction Laboratories (Lexington, KY). A polyclonal antibody to a GST fusion protein containing a region of the β subunit of the murine IL-3 receptor was raised in rabbits and affinity-purified. Immunoprecipitated proteins were resolved on SDS-polyacrylamide gels and electrophoresed transferred to Immobilon membrane (Millipore, Bedford, MA). Immunoblotting was conducted as described using the Enhanced Chemiluminescence Lighting (ECL) system according to manufacturer’s recommendations (Amersham Corp.). All gels were 7% polyacrylamide gels except those used in the analysis of Grb2, which were 12%.

**Phosphatidylinositol Kinase Assay—**Reactions were a modification of a previously described protocol (29, 30). The immunoprecipitated proteins were washed three times with RIPA, twice with PAN (20 mM PIPES (pH 7.0), 20 μM aprotinin, 100 mM NaCl), and resuspended in 50 μl of PAN. A 5-μl aliquot of each sample was removed and placed in a new tube, and 1 μl of 2 mg/ml phosphatidylinositol in 4.5 mM EGTA, 10% Me2SO was added to each reaction. The tubes were incubated at room temperature for 10 min before addition of the reaction mixture containing ATP and incubation at 30 °C for 15 min. The final reaction mixture contained 20 mM HEPES (pH 7.4), 5 mM MgCl2, 0.45 mM EGTA, 10 μM ATP (5 μCi of [γ-32P]ATP), and 0.2 mg/ml PI. Reactions were terminated by the addition of 1 ml of 1 M HCl and extraction of 0.2 ml of CHCl3:methanol (1:1). After discarding the aqueous phase, the organic phase was re-extracted with 1 ml of 2 M phosphatidylinositol in 4.5 mM EGTA, 10% Me2SO and dried at 50 °C.

**RESULTS**

Cbl Is Rapidly Phosphorylated on Tyrosine Residues following IL-3 Stimulation—We have previously described the activation of three Src-like tyrosine kinases following stimulation of 32D c3 cells with IL-3 (11). As part of a study to examine the interaction of Fyn, Hck, and Lyn with the β subunit of the IL-3 receptor, we observed that GST fusion proteins encoding the unique, SH3, and SH2 domains of these three kinases appeared to bind to a tyrosine-phosphorylated protein of approximately 120 kDa. Based upon several recently published studies, we investigated whether this protein might be Cbl since its molecular mass is 120 kDa (14–16, 19, 20, 22, 25). To determine whether this was the case, anti-Cbl immunoprecipitates were immunoblotted with the anti-phosphotyrosine antibody 4G10. The results of this study (Fig. 1), indicate that Cbl is phosphorylated following stimulation of 32D c3 cells with IL-3. A major band of approximately 120 kDa is observed in the anti-Cbl immunoprecipitate immunoblotted with anti-phosphotyrosine antibody. A minor band of 120 kDa can be seen in the anti-phosphotyrosine immunoprecipitate that co-migrates with the tyrosine-phosphorylated band observed in the anti-Cbl immunoprecipitate.

**Fig. 1**. The tyrosine-phosphorylated band of 120 kDa also co-migrates with the phosphotyrosine immunoprecipitate that co-migrates with the tyrosine-phosphorylated band observed in the anti-Cbl immunoprecipitate (Fig. 1, compare lanes 5 and 6). The tyrosine-phosphorylated band of 120 kDa also co-migrates with the 120-kDa band observed in a parallel blot probed with the anti-Cbl antibody (Fig. 1, lanes 5 and 6). The anti-Cbl immunoblot also indicates that only a small portion of the Cbl protein is observed in an anti-Cbl immunoprecipitate (Fig. 1, compare lane 6 with lanes 7 or 8). A comparison of the relative densities of the band in lanes 6–8 indicates that only 12% of the Cbl protein present in anti-Cbl immunoprecipitates is present in the anti-phosphotyrosine immunoprecipitates.

The time course of Cbl phosphorylation was also examined. The results in Fig. 2 suggest that phosphorylation of Cbl can be detected as early as 2 h after IL-3 stimulation and is maximal 20–30 min after stimulation (Fig. 2). This time course was consistently observed in three independent studies. Examination of longer time periods revealed that tyrosine phosphorylation of Cbl could be detected for up to 4 h post-stimulation.
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(data not shown). Immunoblotting of lanes 7–12 with anti-Cbl antiserum indicated that the amount of Cbl protein did not change over the time period examined (data not shown).

Association of Cbl with Grb2—Several previous investigators have noted the association of Cbl with the Grb2 protein (16, 24, 25). This possibility was examined by immunoprecipitation of unstimulated and IL-3-stimulated cell lysates with either anti-Cbl or anti-Grb2 antisera and immunoblot analysis. Immunoblotting the anti-Grb2 immunoprecipitates with anti-Grb2 antisera revealed the expected band of 23 kDa (Fig. 3). The amount of Grb2 present in all lysates did not appear to change following IL-3 stimulation (Fig. 3). Anti-Cbl immunoprecipitates of unstimulated cells contained low amounts Grb2 (Fig. 3). IL-3-stimulation resulted in a 22-fold increase in the amount of Grb2 associated with Cbl (Fig. 3, lane 1 versus lane 2). Densitometry indicates that 27% of Grb2 observed in anti-Grb2 immunoprecipitates was present in the anti-Cbl immunoprecipitates of IL-3-stimulated cells. These results clearly demonstrate the association of a fraction of the total cellular Grb2 with Cbl in unstimulated cells and an increase in that association following IL-3 stimulation.

Association of Cbl with Phosphatidylinositol 3-Kinase—Other investigators have reported the association of Cbl with the p85 subunit of PI 3-kinase (14, 15, 21, 24, 27). This was investigated by immunoprecipitation of unstimulated and IL-3-stimulated cell lysates with anti-phosphotyrosine, anti-Cbl, and anti-PI 3-kinase antibodies, and by immunoblotting with these same antibodies. IL-3 stimulation resulted in the phosphorylation of numerous proteins on tyrosine residues including the β subunit and Cbl; however, tyrosine phosphorylation of the p85 subunit of PI 3-kinase was not observed (Fig. 4, lane 6, top panel). The position of the p85 subunit was determined by reprobing the same blot with anti-p85 antibody. Immunoprecipitation of Cbl was observed with anti-phosphotyrosine antibody in lysates of IL-3-stimulated cells and with anti-Cbl antibody in both unstimulated and stimulated cell lysates (Fig. 4, lanes 1–4, middle panel); however, no Cbl protein was detected in immunoblots of the anti-p85 immunoprecipitates of either unstimulated or stimulated cell lysates (Fig. 4, lanes 5 and 6, middle panel). Consistent with results shown in Fig. 1, Cbl was detected in an anti-phosphotyrosine immunoprecipitate of IL-3-stimulated cells (Fig. 4, lane 2, middle panel). The p85 subunit of PI 3-kinase was detected by immunoblotting with an anti-p85 subunit antibody. Larger amounts of p85 were detected in the anti-p85 immunoprecipitates. There was a clear increase in the amount of p85 detected in anti-phosphotyrosine immunoprecipitates of IL-3-stimulated cells, relative to unstimulated cells. There was a low but detectable amount of p85 in the anti-Cbl immunoprecipitates; however, the amount of p85 in anti-Cbl immunoprecipitates did not increase following IL-3 stimulation (Fig. 4, lanes 5 and 6, bottom panel). The anti-PI 3-kinase

Fig. 1. Cbl is phosphorylated on tyrosine residues following IL-3 stimulation. 32D c3 cells were cultured overnight in the absence of IL-3, then either left unstimulated or stimulated with 100 units/ml recombinant murine IL-3 for 10 min (lanes labeled − and +, respectively). Cells were lysed with RIPA and immunoprecipitated with either anti-phosphotyrosine monoclonal antibody 4G10 (lanes 1, 2, 5, and 6) or anti-Cbl antibody (lanes 3, 4, 7, and 8). Each immunoprecipitate contained lysate prepared from 2 × 10⁷ cells. Following display of the immunoprecipitated proteins on a 7% SDS-polyacrylamide gel, the proteins were transferred to Immobilon and immunoblotted with either anti-Grb2 (Transduction Laboratories catalog no. 616720) or anti-Cbl antibody (lanes 1 and 2). Immunoblotting was with anti-Cbl antibody (lanes 3 and 4), and anti-p85 antibody (lanes 10 and 11). The positions of prestained molecular size markers are indicated on the left side of the figure, and the position of Cbl is indicated on the right side.

Fig. 2. Kinetics of Cbl phosphorylation following IL-3 stimulation. The 32D c3 cells were cultured overnight in the absence of IL-3, then stimulated for 0–30 min with 100 units/ml recombinant murine IL-3. Lysis was with RIPA, and immunoprecipitation was with either 4G10 (lanes 1–6) or anti-Cbl (lanes 7–12). Immunoblotting was with anti-phosphotyrosine antibody 4G10. The time in minutes of stimulation is indicated at the top of each lane. The position of prestained molecular size markers are indicated on the left side of the figure, and the position of Cbl is indicated on the right side.

Fig. 3. Association of Grb2 with Cbl. Unstimulated (−) and stimulated (+) cells were lysed with EB and immunoprecipitated with either anti-Grb2 (Transduction Laboratories catalog no. 616720) or anti-Cbl antibodies. The immunoprecipitated proteins were resolved by gel electrophoresis and subjected to immunoblot analysis with anti-Grb2 antibody. The positions of prestained protein markers are indicated on the left side of the figure, and the positions of the Grb2 is indicated on the right side of the panel.
antibody did co-precipitate tyrosine-phosphorylated proteins with molecular masses of 145, 75, 56, 48, and a broad band at approximately 95–100 kDa from IL-3-stimulated cells (Fig. 4, lane 8, top panel). The 56-kDa band was also observed in the anti-phosphotyrosine immunoprecipitate of IL-3-stimulated cells and most likely represents Shc (data not shown). The identity of 145-kDa band is not the β subunit since it does not react with anti-β antibodies; however, it may represent the recently described SHIP protein, which has been shown to associate with Shc (33–35).

To provide additional evidence that PI 3-kinase associates with Cbl, anti-Cbl immunoprecipitates were assayed for PI 3-kinase activity in comparison to anti-p85 and anti-phosphotyrosine immunoprecipitates. PI 3-kinase activity was present in the anti-phosphotyrosine immunoprecipitates of IL-3-stimulated cells (Fig. 5, lanes 1–4). The amount of kinase activity in anti-phosphotyrosine immunoprecipitates increased with the length of stimulation and appeared to plateau after 10–20 min (Fig. 5 and data not shown). PI 3-kinase activity was also present in anti-p85 immunoprecipitates at all time points examined (Fig. 5, lanes 9–12). PI kinase activity was detectable in anti-Cbl immunoprecipitates (Fig. 5, lanes 7 and 8). The PI kinase activity present in anti-Cbl immunoprecipitates appears to peak at 5 min and declines by 20 min after IL-3 stimulation. It is clear from the data in Fig. 5 that the amount of PI kinase activity present in anti-Cbl immunoprecipitates is dramatically less than that present in either the anti-phosphotyrosine or anti-p85 immunoprecipitates. PhosphorImager analysis quantitation of the spots corresponding to PIP in Fig. 5 reveals that at the peak time point, the amount of kinase activity in anti-Cbl immunoprecipitates corresponded to 10% of the amount observed in anti-p85 immunoprecipitates or 10% of the amount activity observed at 20 min in anti-phosphotyrosine immunoprecipitates.

Association of Cbl with the Fyn Tyrosine Kinase—We have previously described the activation of multiple Src-like kinases (Fyn, Hck, and Lyn), following stimulation of 32D cl3 cells with IL-3 (11). The association of Cbl with Src-like kinases has been described by other investigators (15, 19, 20). These observations prompted us to determine whether Cbl was associated with Fyn, Hck, or Lyn in 32D cl3 cells, and whether this association was altered following cytokine stimulation. Lysates of unstimulated and IL-3-stimulated cells were immunoprecipitated with anti-Cbl antibody and immunoblotted with anti-Fyn monoclonal antibody. Cbl was observed to co-precipitate Fyn from lysates of both unstimulated and IL-3-stimulated cells (Fig. 6). There was no apparent change in the amount of Fyn associated with Cbl following IL-3 stimulation (Fig. 6). Similar immunoblotting studies conducted with anti-Hck and anti-Lyn did not reveal association of these kinases with Cbl (data not shown).

Phosphotyrosine-independent Binding of Cbl to Bacterial Fusion Proteins Encoding the unique, SH3, and SH2 Domains of Src-like Kinases—As mentioned above, the tyrosine phosphorylation of a 120-kDa protein in IL-3-stimulated 32D cl3 cells was first detected in binding studies utilizing GST fusion proteins containing the unique, SH3, and SH2 domains of Fyn, Hck, or Lyn. Fig. 7 shows the results of one such study, in which the binding of Cbl from lysates of IL-3-stimulated 32D cl3 cells to GST-FYN and GST-HCK is examined. No binding of Cbl was observed using GST (Fig. 7, lanes 1 and 2). GST-FYN bound to Cbl in lysates of either unstimulated and IL-3-stimulated cells (Figs. 7, lanes 3 and 4). There was no difference in the amount of Cbl bound to GST-FYN when unstimulated or stimulated cell extracts were used in the binding assays, and no detectable tyrosine-phosphorylated Cbl was detected in unstimulated cells (Fig. 7, lanes 3 and 4, top and bottom panels). These results suggested that the binding of Cbl to GST-FYN might be largely phosphotyrosine-independent. GST-HCK was also observed to bind to Cbl in lysates of both unstimulated and IL-3-stimulated cells (Fig. 7, lanes 9 and 10). There did appear to be a 2-fold increase in the amount of Cbl that bound to

**Association of PI 3-kinase activity with Cbl.** 32D cl3 cells were stimulated with IL-3 for 0–20 min, and cell lysates were immunoprecipitated with anti-phosphotyrosine antibody 4G10 (lanes 1–4), anti-Cbl antibody (lanes 5–8), anti-p85 subunit of PI3'-kinase (Transduction Laboratories catalogue no. PI3030) (lanes 9–12), or nonimmune rabbit serum (NRS, lanes 13). The immunoprecipitated proteins were used in a PI kinase assay. The time in minutes of stimulation and the antibodies used for immunoprecipitation are indicated at the bottom of the figure. The position of phosphatidylinositol phosphate (PIP) is indicated by the arrow on the right side of the panel. Lane numbers are indicated across the top of the panel.
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Binding assays were conducted as described in Fig. 3 with GST-FYN (lanes 1–3), GST-HCK (lanes 4–6), and GST-LYN (lanes 7–9). Each cell lysate contained 2 × 10^7 cells and 2 nmol of GST fusion protein in 1.5 ml. 30 mM phosphoserine was added to lanes 2, 5, and 8, while 30 mM phosphotyrosine was added to lanes 3, 6, and 9. Immunoblot analysis was with anti-Cbl antiserum.

were involved in binding to Cbl, we used different GST fusion proteins containing all or part of a specific domain of either Fyn or Hck and examined their binding to Cbl by anti-Cbl immunoblotting or to tyrosine-phosphorylated proteins by anti-phosphotyrosine immunoblotting. GST-FYN-SH2 bound to Cbl present in both unstimulated and stimulated cells (Fig. 7, lanes 7 and 8); however, only the Cbl in IL-3-stimulated cells was tyrosine-phosphorylated. No binding of Cbl was observed to the GST-FYN-unique (Fig. 7, lanes 5 and 6) or to GST-FYN-SH3 (data not shown). When GST-FYN-SH2 was used in the binding assay, there was a 2-fold increase in the amount of Cbl that bound when lysates of IL-3-stimulated cells were used compared to the amount that bound when lysates of unstimulated cells were used.

In contrast to the results obtained with single domains of Fyn, no binding to Cbl was observed when GST fusion proteins containing either the unique, the SH3, or the SH2 domains of Hck were used (Fig. 7, lanes 11–16). This suggests that binding of Hck to Cbl may involve the interactions of multiple domains to generate a stable complex. The large band migrating immediately above Cbl in the anti-phosphotyrosine blot includes the tyrosine-phosphorylated β subunit of the IL-3 receptor. Identity of this band was confirmed by immunoblotting with an anti-β subunit antibody (data not shown).

The data in Fig. 7 clearly suggests that Fyn and Cbl may associate, via the SH2 domain of Fyn, in phosphotyrosine-independent manner. As another means to determine whether this binding was phosphotyrosine-independent, either 30 mM phosphoserine or 30 mM phosphotyrosine was added to binding reactions involving GST-FYN, GST-HCK, or GST-LYN (Fig. 8). In each of these cases, we did not observe a decrease in the amount of Cbl bound to GST-FYN, GST-HCK, or GST-LYN when either phosphoserine or phosphotyrosine was present (Fig. 8). These studies suggest to us that at least a portion of this binding must be mediated by phosphotyrosine-independent interactions.

**DISCUSSION**

In this paper we have described the phosphorylation of Cbl on tyrosine following stimulation of factor-dependent 32D c3 cells with IL-3. Association of Cbl with three other molecules involved in signal transduction was noted; Grb2, Fyn, and the p85 subunit of PI 3-kinase. IL-3 stimulation increased the amount of Grb2 associated with Cbl; however, it did not appear to alter the amount of Fyn or p85 associated with Cbl. Activated PI kinase activity was associated with Cbl following IL-3 stimulation; however, the Cbl-associated activity was only 10% of the activity noted in anti-phosphotyrosine or anti-p85 immunoprecipitates. The association of Cbl with Src-like kinases, the p85 subunit of PI 3-kinase, or PI kinase activity following stimulation of cytokine family receptors has not been described before and is novel to this report. Our results are consistent

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**Fig. 6. Association of Cbl with Fyn in unstimulated and IL-3-stimulated cells.** Lysates of unstimulated (−) and IL-3-stimulated (+) 32D c3 cells were immunoprecipitated with anti-Cbl antibody, and the immunoprecipitated proteins resolved by SDS-gel electrophoresis and subjected to immunoblot analysis with anti-Fyn monoclonal antibody Transduction Labs catalogue no. F19720. The position of Fyn was determined by inclusion of a whole cell lysate (lane marked WCL) as a positive control. The positions of prestained protein markers are indicated on the left side of the panel, and the position of Fyn is indicated on the right.

**Fig. 7. Binding of Cbl to specific domains of Fyn and Hck.** Binding assays were conducted with lysates of unstimulated and stimulated cells as described in Fig. 3 with the indicated GST fusion proteins. Binding assays contained GST-FYN (lanes 1 and 2), GST-FYN-unique (amino acids 1–22; lanes 3 and 4), GST-FYN-SH3 (lanes 5 and 6), GST-FYN-SH2 (lanes 7 and 8), GST-HCK (lanes 9 and 10), GST-HCK-unique (lanes 11 and 12), GST-HCK-SH3 (lanes 13 and 14), and GST-HCK-SH2 (lanes 15 and 16). Lane 17 contains an anti-Cbl immunoprecipitate of unstimulated cells. Immunoblotting was with anti-Cbl antiserum. Immunoblotting in the top panel was with anti-phosphotyrosine antibody. The filter was then stripped and reprobed with an anti-Cbl antibody (bottom panel). The positions of prestained protein markers are indicated on the left side of the top panel. The positions of the β subunit and Cbl are indicated on the right side of each panel.

GST-HCK when lysates of IL-3-stimulated cells were used, compared to the amount of Cbl bound when lysates of unstimulated cells were used. Consistent with above observations using GST-FYN, tyrosine phosphorylation of Cbl was not observed in lysates of unstimulated cells.

Phosphotyrosine-dependent interactions most likely involve SH2 domains (36–38), while phosphotyrosine-independent binding would be expected to involve either the unique or SH3 domain, or both. To determine what region(s) of Fyn and Hck.
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with a previous publication demonstrating tyrosine phosphorylation of Cbl following stimulation of factor-dependent cells with erythropoietin or GM-CSF (25). In that work, the constitutive association of Grb2 with Cbl, via the SH3 domain of Grb2, was noted.

Using GST fusion proteins in binding assays, we have determined that the SH2 domain of Fyn can bind to Cbl in a phosphotyrosine-independent manner. Observations supporting this conclusion include: (a) GST-FYN-SH2 bound to non-phosphorylated Cbl, and (b) 30 m\textmu{}M phosphotyrosine did not block this binding. While several other investigators have demonstrated that binding of proteins to Cbl is mediated by SH2 domains, none have investigated whether these interactions are phosphotyrosine-dependent. The phosphotyrosine-independent binding of the v-Abl SH2 domain to Src has been described (39). In addition, a 62-kDa protein has been described that binds the SH2 domain of Lck in a phosphotyrosine-independent manner (40, 41). The possibility that these binding interactions represent the binding of SH2 domains to phospholipid-modified proteins remains to be investigated (42).

Activation of PI 3-kinase by cytokines such as IL-3 and GM-CSF has been reported by several investigators (43–45). Although Wang et al. (43) noted the low level tyrosine-phosphorylation of p85, other investigators, including this report, have not observed phosphorylation of p85 following stimulation with IL-3 or GM-CSF (45, 46). The fact that PI 3-kinase activity is detected in anti-phosphotyrosine immunoprecipitates (this report and Ref. 45), suggests that this enzyme is complexed to other tyrosine-phosphorylated proteins. Candidate tyrosine-phosphorylated proteins to which p85/PI 3-kinase might asso-

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