p120cbl Is a Major Substrate of Tyrosine Phosphorylation upon B Cell Antigen Receptor Stimulation and Interacts in Vivo with Fyn and Syk Tyrosine Kinases, Grb2 and Shc Adaptors, and the p85 Subunit of Phosphatidylinositol 3-Kinase*

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We and others have demonstrated that the c-bcl proto-oncogene product is one of the earliest targets of tyrosine phosphorylation upon T cell receptor stimulation. Given the similarities in the B and T lymphocyte antigen receptors, and the induction of pre-B leukemias in mice by the v-cbl oncogene, we examined the potential involvement of Cbl in B cell receptor signaling. We demonstrate prominent and early tyrosine phosphorylation of Cbl upon stimulation of human B cell lines through surface IgM. Cbl was associated in vivo with Fyn and, to a lesser extent, other Src family kinases. B cell activation also induced a prominent association of Cbl with Syk tyrosine kinase. A substantial fraction of Cbl was constitutively associated with Grb2 and this interaction was mediated by Grb2 SH3 domains. Tyrosine-phosphorylated Shc, which prominently associated with Grb2, was detected in association with Cbl in activated B cells. Thus, Grb2 and Shc adaptors, which associate with immunoreceptor tyrosine-based activation motifs, may link Cbl to the B cell receptor. B cell activation also induced a prominent association between Cbl and the p85 subunit of phosphatidylinositol (PI) 3-kinase resulting in the association of a substantial fraction of PI 3-kinase activity with Cbl. Thus, Cbl is likely to play an important role to couple the B cell receptor to the PI 3-kinase pathway. Our results strongly suggest a role for p120cbl in signaling downstream of the B cell receptor and support the idea that Cbl participates in a general signal transduction function downstream of the immune cell surface receptors.

(TCR)2 or B cell receptor (BCR)) leads to a cascade of biochemical events that culminates in lymphocyte activation (1). The T and B cell antigen receptors signal through associated CD3/TCR (ζ/η) and the Igα/β chains, respectively (1, 2). The cytoplasmic tails of these receptor-associated polypeptides contain conserved sequence motifs, the immunoreceptor tyrosine-based activation motifs (I-TAMs), which are necessary and sufficient for coupling to downstream signaling machinery (1, 2). An early and an obligatory step in lymphocyte activation is the tyrosine phosphorylation of the I-TAMs and a number of cellular substrates (1, 2). However, lymphocyte antigen receptors and their associated chains lack intrinsic tyrosine kinase domains, indicating the role of non-receptor protein tyrosine kinases (PTKs). Indeed, the CD3/ζ chains of the TCR and the CD4/8 co-receptors associate with the Src-family PTKs Fyn and Lck, respectively (3–6). Similarly, Igα/Igβ chains interact with the Src family kinases Lyn, Blk, Fyn, and Lck in B cells (7–9). Furthermore, upon tyrosine phosphorylation by the Src family kinases, the I-TAMs recruit the Syk/ZAP-70 PTKs in an activation-dependent manner (10–14). Genetic and biochemical data have clearly shown that Src family and ZAP-70/Syk PTKs are crucial for lymphocyte activation (15–22). Therefore, identification and characterization of the early targets of receptor-associated PTKs has become a major focus of studies aimed at elucidating the mechanisms of lymphocyte signaling.

Many of the identified effectors that function downstream of the PTKs in T and B cells are shared; these include phospholipase C-γ1, phosphatidylinositol (PI) 3-kinase, adapter proteins Shc and Grb2 that couple receptors to Ras pathway, and the proto-oncogene product Vav (1, 2). Thus, additional effectors are also likely to be shared between T and B cells.

Recently, we identified a Fyn/Lck SH3 domain-binding protein, p120cbl, which was present as preformed complexes with Fyn and Lck in unstimulated T cells and was rapidly tyrosine phosphorylated upon TCR stimulation (23, 24). Subsequently, we and others have demonstrated this polypeptide to be the product of the c-bcl proto-oncogene (25, 26). c-bcl is the cellular homolog of v-bcl, the oncogene of the Cas NS-1 murine retrovirus.

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1 The abbreviations used are: TCR, T cell antigen receptor; BCR, B cell antigen receptor; PTK, protein tyrosine kinase; pY, phosphoryltyrosyl; PI, phosphatidylinositol; pS, phosphoserine; PIP2, phosphatidylinositol 4,5-bisphosphate; CD, cluster of differentiation.
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Materials and Methods

Antibodies—The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): rabbit anti-p120<sup>cbl</sup> (sc-170), rabbit anti-Grb2 (sc-255), rabbit anti-Fyn (sc-16), rabbit anti-Lyn (sc-15), rabbit anti-Blk (sc-329), mouse anti-Lck mAb (sc-433), and rabbit anti-Syk (sc-573). The following antibodies were from Transduction Laboratories (Lexington, KY): rabbit anti-Shc (Sc-14630) and rabbit anti-p110<sub>2</sub>-SHC (P13030), rabbit anti-human Ig (62-7500) was from Zymed. 4G10 (anti-pY；lgG2a) has been described (38). Normal rabbit serum was used as a negative control.

Glutathione S-Transferase (GST) Fusion Proteins—Wild-type murine Grb2, Grb2<sup>SH2</sup>, p85<sub>a</sub> mutant (R86K), and Grb2<sup>β</sup> with mutations in both SH3 domains (P49L,P206L) were expressed in pGEX-3X vector (Pharmacia), as described (25). The Grb2<sup>SH2</sup> mutant (R86K) is depicted as 3–2–3, whereas Grb2<sup>β</sup> with mutations in both SH3 domains (P49L,P206L) is depicted as 3’–2–3’. Fusion proteins were affinity purified on glutathione-Sepharose beads (Pharmacia Biotech Inc.) and quantified, as described (23, 24).

Cells—Surface IgM-expressing human B cell lines Ramos, Daudi, and Raji were grown in RPMI 1640 supplemented with 20% heat inactivated FCS, 50 U/ml penicillin, and 50 mg/ml streptomycin, and 2% fetal calf serum (Hyclone). 7% fetal calf serum (Hyclone).

Activation of B Cell Lines with Anti-IgM—Cells were washed and resuspended in RPMI 1640 with 20% HEPES at 10<sup>6</sup>cells/ml and incubated at 37°C for 2 min. Rabbit anti-human IgM was added to a final concentration of 20 μg/ml and cells were incubated further for 2 min or the indicated times. Cells were directly lysed by adding 3 volumes of cold lysis buffer (0.5% Triton X-100, 150 mm NaCl, 10% glycerol, 1% NP-40, 50 mm Tris, pH 7.5, 100 mm sodium chloride, 1 mm EDTA, 1 mm phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and pepstatin, 1 mm vanadate, and 10 mm sodium fluoride).

Immunoprecipitation, Gel Electrophoresis, and Immunoblotting of Proteins—Cell lysates prepaid with Staphylococcus aureus Cowan I strain (Pansorbin, Calbiochem) were incubated with antibodies for 1–2 h at 4°C, and 20 μl of Protein A-Sepharose 4B (Pharmacia) was added for an additional 45–60 min. Bound proteins were eluted from washed beads by boiling in Laemmli sample buffer with 2-mercaptoethanol, resolved by SDS-PAGE, transferred to PVDF membranes (Immobil-P, Millipore, Bedford, MA), and subjected to immunoblotting, as described (25), using horseradish peroxidase-conjugated Protein A (Cappel-Organon Technika, Durham, NC), followed by enhanced chemiluminescence (ECL) detection according to manufacturer's recommendations (DuPont NEN). Reprobing of membranes was as described (25).

Measurement of PI 3-Kinase Activity—The immunoprecipitates (see above) were washed four times in phosphate-buffered saline (137 mm NaCl, 15.7 mm Na<sub>2</sub>HPO<sub>4</sub>, 1.47 mm KH<sub>2</sub>PO<sub>4</sub>, 2.68 mm KCl, pH 7.4) plus 1% (v/v) Nonidet P-40, followed by three washes in TNE (10 mm Tris, pH 7.5, 100 mm NaCl, 1 mm EDTA). All wash solutions contained 0.2 mm sodium orthovanadate. To assay the lipid kinase activity, PI (Avanti Polar Lipids; left panel in Fig. 6) or phosphatididylinositol 4,5-bisphosphate (PIP<sub>2</sub>, Sigma; right panel in Fig. 6) plus [γ<sup>32</sup>P]<sub>ATP</sub> (10 μCi/sample) were added to the immunoprecipitates for 10 min at room temperature. The phosphoinositides were suspended in 10 mm HEPES, pH 7.5, 100 mm NaCl, 1 mm EDTA. All wash solutions contained 0.2 mm sodium orthovanadate. For the TLC identification of PIP<sub>2</sub>, a chloroform:methanol mixture was used at a 60:47:11.3:2 ratio. For the identification of PIP<sub>2</sub>, a 1-propanol, 2 M acetic acid solvent system was used at a 65:35 ratio. Lipid species were visualized by autoradiography.

RESULTS

Cbl is a Major Tyrosine Phosphorylation Substrate upon BCR Stimulation—To investigate the involvement of p120<sup>cbl</sup> in BCR signaling, we utilized surface IgM-expressing human B cell lines Ramos, Daudi, and Raji. The BCR on these cells was cross-linked with anti-IgM antibody for various time points, and their whole cell lysates or anti-Cbl immunoprecipitations were subjected to SDS-PAGE, followed by anti-pY immunoblotting.

Relatively low levels of tyrosine phosphorylation on various polypeptides were seen in unstimulated cell lysates harvested at either 2 or 30 min of incubation at 37°C (Fig. 1, lanes 1 and 2). Anti-IgM stimulation led to a time-dependent increase in tyrosine phosphorylation of a number of cellular polypeptides, including a prominent 120-kDa phosphoprotein. Phosphorylation of this polypeptide was observed at the earliest time point tested (10 s), reached a maximum by 1–2 min, and declined slightly thereafter with substantial phosphorylation still detectable at 60 min (Fig. 1, lanes 3–9, and data not shown). Anti-pY immunoblotting of anti-Cbl immunoprecipitates showed that the 120-kDa pY<sup>-</sup> polypeptide observed in lysates copurified with Cbl (Fig. 1, right panel, lanes 10–18). Low basal phosphorylation of Cbl was observed in the absence of anti-IgM stimulation (lanes 10 and 11). Notably, directly im-
mammunoprecipitated Cbl was rapidly and prominently tyrosine phosphorylated with a kinetics identical to that observed for munoprecipitated Cbl was rapidly and prominently tyrosine phosphorylation in two other B cell lines, Daudi and Raji. In addition to Ramos, we also examined anti-IgM-induced Cbl phosphorylation in two other B cell lines, Daudi and Raji. In each case, anti-pY immunoblotting of the immunoprecipitated Cbl showed prominent tyrosine phosphorylation when examined at 2 min after stimulation (Fig. 2, lanes 3 and 4 in each panel). Similarly, anti-Cbl immunoblotting showed that Cbl was prominently immunoprecipitated by anti-pY antibody from lysates of the anti-IgM-activated but not the unstimulated cells (Fig. 2, lanes 5 and 6, in each panel). These results demonstrate that Cbl tyrosine phosphorylation is not restricted to Ramos B cells, but is a general feature of B cells that carry a surface IgM receptor.

Association of Cbl with BCR-associated Tyrosine Kinases—The potential signal transduction function of p120

Cbl was first revealed by its ability to interact with SH3 and SH2 domains of the T cell Src family PTKs (23, 24), and subsequent analyses in myeloid and T cells demonstrated these associations in vivo (25, 36). Since Src family PTKs also mediate initial tyrosine phosphorylation reactions upon BCR triggering (7–9), we wished to examine whether Cbl interacts with these kinases in B cells. In addition, we examined the interaction of Cbl with Syk PTK, which represents an essential downstream mediator of tyrosine phosphorylation in B cells (13, 14) and has been observed to associate with Cbl after FcγR triggering of myeloid cells (36). For this purpose, anti-PTK immunoprecipitates from unstimulated or anti-IgM-stimulated Ramos B cells were subjected to anti-pY and anti-Cbl immunoblotting.

A small fraction of Cbl was readily detectable in association with Fyn prior to anti-IgM activation of Ramos cells and this association increased considerably following stimulation (Fig. 3, anti-Cbl blot, lanes 9 and 10). Anti-pY immunoblotting showed that Fyn-associated Cbl underwent an activation-dependent tyrosine phosphorylation. A much smaller amount of Cbl was observed in anti-B1k immunoprecipitates from activated Ramos cells (anti-Cbl blot, lanes 5 and 6), and activation-dependent tyrosine phosphorylation of Blk-associated Cbl was also observed upon longer exposures. Much smaller amounts of Cbl were detected in association with Lyn and Lck and could be visualized primarily in anti-pY immunoblot upon longer exposure (not shown). Thus, Cbl associates detectably with some B cell Src-family PTKs (Fyn and Blk), but poorly with others (Lyn and Lck). Under the conditions of the experiment, all antibodies were able to immunoprecipitate their respective polypeptides, as determined by direct immunoblotting (data not shown); these polypeptides are visible in anti-pY immunoblotting of anti-Lyn, anti-Lck, and anti-Fyn immunoprecipitates just above the Ig heavy chain bands (Lck resolves as a doublet) (Fig. 3, lanes 3, 4, and 7–10). Low stoichiometry of association, together with comigrating Ig heavy chains made it difficult to use immunoblotting with antibodies to Src family PTKs to demonstrate their association with Cbl (data not shown).

Anti-pY immunoblotting of anti-Cbl immunoprecipitates showed several associated polypeptides migrating between Ig and Cbl bands as well as other higher molecular weight species (Fig. 3, lanes 11 and 12). A Cbl-associated 70 kDa polypeptide comigrated with Syk (compare lanes 11 and 12 with 13 and 14) and anti-Syk immunoblotting confirmed its identity (data not shown). Anti-Syk immunoprecipitates revealed a prominent 120-kDa phosphotyrosyl polypeptide comigrating with Cbl; the phosphotyrosine content of this band showed an activation-dependent increase (Fig. 3, lanes 13 and 14). Anti-Cbl immunoblot confirmed that Cbl was associated with Syk in an activation-dependent manner. Thus, Syk and Cbl show a prominent activation-dependent association in B cells. A 70-kDa tyrosyl phosphoprotein comigrating with Syk was also observed to associate with Src kinases, in particular with Lck, in an activation-dependent manner (Fig. 3, lanes 3–10); additional unidentified pY polypeptides were also noted in anti-Lck

Fig. 1. Cross-linking of B cell antigen receptor on the Ramos human B cell line induces rapid and sustained tyrosine phosphorylation of p120

C. Cells were washed and resuspended in serum-free medium, warmed to 37°C, and then incubated for the indicated times (seconds or minutes) either in the absence (−) or in the presence (+) of 20 μg/ml rabbit anti-human IgM. Cells were lysed in Triton X-100 lysis buffer, and precleared lysates were resolved on SDS-9% PAGE either as such (Lysate, left panel; 10⁶ cells per lane) or after anti-Cbl immunoprecipitation (α-cbl i.p., right panel; 5 × 10⁷ cells/lane). Resolved polypeptides were transferred to PVDF membrane and incubated with anti-pY antibody followed by Protein A-horseradish peroxidase (upper panels). Blots were developed using the ECL method. Filters were then stripped and re-immunoblotted with anti-Cbl antibody to demonstrate equal loading in all lanes (lower panels). Cbl and Ig (heavy chain of the immunoprecipitating antibody) are indicated on the left. All lanes within each blot are from a single exposure.
and anti-Blk immunoprecipitates (lanes 5–8).

To further elucidate the role of Cbl in B cell signaling, we examined its ability to form in vivo complexes with various SH2 and SH3 domain-containing proteins, which we and others have shown to interact with Cbl in T lymphocytes and other cells (25, 32–34). For this purpose, immunoprecipitations were carried out with various antibodies using lysates of unstimulated or anti-IgM-stimulated Ramos cells, and these were subjected to anti-pY immunoblotting and then reprobed with immunoprecipitating antibodies.

As expected from the above studies, anti-pY immunoblotting showed activation-dependent tyrosine phosphorylation of Cbl (Fig. 4, lanes 3 and 4, both bands of the doublet are specific), whereas anti-Cbl immunoblotting revealed activation-dependent immunoprecipitation of Cbl by anti-pY antibody (lanes 11 and 12). Anti-Cbl immunoblotting revealed that a substantial fraction of this polypeptide co-immunoprecipitated with Grb2 when lysates of unstimulated Ramos cells were analyzed (Fig. 4, lane 5). This was confirmed by anti-Grb2 immunoblotting of anti-Cbl immunoprecipitate (lane 3). Cbl-Grb2 association was relatively unaltered after B cell activation (Fig. 4, lanes 3–6); in some experiments a modest increase in association was noted (data not shown). In keeping with the association of a substantial fraction of Cbl with Grb2, anti-Cbl antibody coimmunoprecipitated small amounts of a number of phosphotyrosyl polypeptides which represented major Grb2-associated phosphotyrosyl proteins in activated Ramos cells (Fig. 4, anti-pY blot, compare lanes 4 and 6). These included a 52-kDa polypeptide comigrating with Shc (lane 8), a 145-kDa polypeptide previously observed in association with Shc in B cells (40, 41) (see lane 8), and several others migrating at 70–100 kDa. Association between Shc and Cbl was directly confirmed by anti-Shc blotting which revealed Shc in anti-Cbl immunoprecipitate of activated cells (Fig. 4, lane 4); anti-Cbl antibody also detected a

**FIG. 2.** Tyrosine phosphorylation of Cbl by BCR cross-linking in Daudi and Raji human B cell lines. Immunoprecipitations were carried out with the indicated antibodies (I.P. Abs) from Triton X-100 lysates of $5 	imes 10^9$ unstimulated (−) or anti-IgM-stimulated (+) Daudi (left) or Raji cells (right). Immunoprecipitates were resolved on SDS-9% PAGE, transferred to PVDF membrane, and subjected to serial immunoblotting with anti-pY (top) and anti-Cbl (bottom) antibodies. NRS, normal rabbit serum, used as a negative control. A constitutively phosphorylated polypeptide co-migrating with Cbl is immunoprecipitated by anti-pY antibody from unstimulated Daudi cells; however, it is unreactive with anti-cbl antibody. All lanes within each blot are from a single exposure.

**FIG. 3.** In vivo association of Cbl with B cell tyrosine kinases. Immunoprecipitations were carried out with the indicated antibodies (I.P. Abs, shown on top) from Triton X-100 lysates of $5 	imes 10^9$ unstimulated (−) or anti-igM-stimulated (+) Ramos cells. Immunoprecipitates or whole cell lysates (10^6 cells) were resolved on SDS-9% PAGE, transferred to PVDF membrane, and subjected to anti-pY immunoblotting (top panel). The membrane was stripped and reprobed with anti-cbl antibody (lower panel). Immunoprecipitated species and Ig are indicated on the left. Bands corresponding to Lyn, Lck (a doublet), and Fyn are seen immediately above the Ig band. Blk bands co-migrate with Ig. In lane 6 of the anti-pY immunoblot (anti-Blk), Cbl migrates immediately above a major background band which is seen in lanes 5 and 6. All lanes within each blot are from a single exposure.

**FIG. 4.** In vivo association of p120^{cbl} with Grb2, Shc, and p85 subunit of the PI 3-kinase in Ramos B cells. Immunoprecipitations from Triton X-100 lysates of $5 	imes 10^9$ unstimulated (−) or anti-igM-stimulated (+) Ramos cells were carried out with the indicated antibodies (I.P. Abs, shown on top). Immunoprecipitates or whole cell lysates (from 10^6 cells) were resolved on SDS-9% PAGE, transferred to PVDF membrane, and subjected to serial immunoblotting with antibodies indicated on the right. Immunoprecipitated species are indicated by arrows or brackets; Ig, immunoglobulin heavy chain. Each immunoblot represents a reprobing of the appropriate parts of the filter shown on top. All lanes within each blot are from a single exposure.
small amount of Cbl in anti-Shc immunoprecipitate upon longer exposure of the blot (not shown). Concurrently, a tyrosine-phosphorylated 120-kDa species comigrating with Cbl was observed in anti-Shc immunoprecipitate (lane 8). As expected, a prominent association between Grb2 and Shc was detected by anti-Grb2 immunoblotting of anti-Shc immunoprecipitates (lanes 7 and 8) as well as by anti-Shc and anti-pY immunoblotting of anti-Grb2 immunoprecipitates (lanes 5 and 6).

Anti-pY immunoblotting of anti-PI 3-kinase p85 immunoprecipitates (Fig. 4, lanes 9 and 10) revealed the activation-dependent appearance of major pY polypeptides migrating at 120 and 100 kDa. The 100-kDa polypeptide is likely to be the CD19, previously shown to be tyrosine phosphorylated upon B cell activation and to interact with the 3-kinase p85 (42). The 120-kDa pY polypeptide comigrated with Cbl. Anti-Cbl immunoblotting of PI 3-kinase p85 immunoprecipitates (Fig. 4, lanes 9 and 10) and anti-PI 3-kinase p85 blotting of anti-Cbl immunoprecipitates (Fig. 4, lanes 3 and 4) demonstrated that a substantial fraction of Cbl became associated with PI 3-kinase p85 after anti-IGM activation of Ramos cells, whereas the association was undetectable in unstimulated cells. The amount of PI 3-kinase p85 polypeptide detected in anti-Cbl immunoprecipitates was nearly as much as in anti-pY immunoprecipitates (Fig. 4, compare lanes 4 and 12). Concurrently, small amounts of Grb2 and Shc also became associated with PI 3-kinase p85, suggesting that larger order complexes of Grb2, Cbl, Shc, and PI 3-kinase p85 are induced by B cell activation. Consistent with this suggestion, all of these polypeptides were detected in anti-pY immunoprecipitates from activated cells (lane 12), even though Grb2 and PI 3-kinase p85 were not tyrosine phosphorylated (Fig. 6 and data not shown).

Interaction between Cbl and Grb2 Is Exclusively Mediated Through Grb2 SH3 Domains—Although we have demonstrated that Grb2-Cbl interaction in T lymphocytes was exclusively mediated by Grb2 SH3 domains (25), others have suggested an additional role for the Grb2 SH2 domain (32). To directly assess the nature of Grb2-Cbl association in B cells, in vitro binding analyses were carried out using GST fusion proteins of Grb2 carrying mutations in the ligand-binding pockets of the SH2 or SH3 domains. Lysates of unstimulated or anti-IgM-stimulated Ramos cells were incubated with bead-immo-

**Fig. 5.** Binding of p120<sup>Grb2</sup> to GST fusion proteins of Grb2 is exclusively mediated through Grb2 SH3 domains. Cell lysates from 1.5 × 10<sup>7</sup> unstimulated cells (−) or cells stimulated with anti-IgM antibody for 2 min at 37 °C (+) were incubated for 1 h at 4 °C with GST fusion proteins noncovalently immobilized on glutathione-Sepharose beads (5 μl of packed beads; 10 μg of fusion protein; total volume 1 ml), and bound proteins were solubilized in sample buffer. Binding reactions or whole cell lysate (from 10<sup>8</sup> cells) were resolved by SDS-PAGE and subjected to anti-Cbl immunoblotting (upper panel) using Protein A-horseradish peroxidase conjugate and ECL detection. The filter was stripped and immunoblotted with anti-pY antibody (lower panel), w.t., wild type Grb2. 3–2–3 refers to NH<sub>2</sub>-terminal SH3, SH2, and C-terminal SH3 domains of Grb2. Asterisks denote mutated domains. Mutated residues were: NH<sub>2</sub>-terminal SH3, P49L; SH2, R86K; COOH-terminal SH3, P206L. Cbl and Shc are indicated on the left. α cbl i.p. (lanes 9–10), anti-Cbl immunoprecipitation from the same amount of cell lysate as used for binding reactions.
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Fig. 6. BCR activation does not induce detectable tyrosine phosphorylation of the p85 and p110 subunits of PI 3-kinase. Anti-PI-3 kinase p85 immunoprecipitations from 2 x 10^7 unstimulated Ramos cells (–) or from cells stimulated with anti-IgM for 2 min (+) were subjected to serial immunoblotting with antibodies shown on top. Arrows point to p110 (Cbl), the p110 and p85 subunits of PI 3-kinase, and Ig heavy chain (Ig). Overlay of autoradiograms confirmed that p85 and p110 did not correspond to any of the major phosphotyrosyl polypeptides.

Since p110 subunit migrated close to Cbl, lack of tyrosine phosphorylation on p110 was independently confirmed using cell lysates that had been denatured by boiling in the presence of SDS (data not shown).

Having established that the PI 3-kinase subunits are not tyrosine phosphorylated, we examined the relative role of Cbl in recruiting PI 3-kinase activity into phosphotyrosyl signaling complexes. For this purpose, we measured the lipid kinase activity associated with Cbl immunoprecipitates from unstimulated and anti-IgM-stimulated Ramos cells. As shown in Fig. 7 (upper left panel), a substantial fraction of PI-kinase activity was observed in anti-Cbl immunoprecipitates of activated Ramos cells when compared to the activity present in anti-PI-3-kinase p85 immunoprecipitates. Further analysis of the Cbl-associated lipid kinase activity revealed that it contained specific PI 3-kinase activity (Fig. 7, upper right panel). Significantly, the level of PI-kinase activity in anti-Cbl and anti-phosphotyrosyl immunoprecipitates was comparable (Fig. 7, upper left panel, lane 4 versus 6), suggesting that Cbl may represent a predominant mechanism to recruit PI 3-kinase activity into phosphotyrosyl complexes. In each experiment, anti-PI-3-kinase p85 immunoblot of the same samples showed that the level of lipid kinase activity correlated well with the amount of p85 PI 3-kinase protein (Fig. 6, bottom panels).

DISCUSSION

The potential signal transduction role of the c-bcr proto-oncogene product was revealed by its cloning as an NCK SH3-domain-binding protein (35), a prominent tyrosine phosphorylation of Cbl polypeptide upon triggering through the TCR (26), and identification of the Fyn/Lck SH3 domain-binding T-cell phosphoprotein p120 as Cbl (23-25). Given the strong similarities between T and BCR signaling, it appeared likely that Cbl may also participate in signaling downstream of the BCR. Here, we used several human B cell lines that have been widely used to study BCR signaling to demonstrate that Cbl is a prominent and early substrate of tyrosine phosphorylation upon triggering through the B cell antigen receptor. Recently, Cbl was also shown to undergo rapid tyrosine phosphorylation upon triggering through Fcγ receptor (36, 37) as well as the granulocyte macrophage colony stimulating factor and erythropoietin receptors (33), which also signal through non-receptor PTKs. Thus, it is likely that Cbl plays a general role in signaling through hematopoietic cell surface receptors. However, Cbl phosphorylation was not observed upon triggering through FcεR, which also utilizes Src family kinases and Syk (36), suggesting that the role of Cbl downstream of the cell surface receptors is somewhat selective.

Cbl phosphorylation upon BCR stimulation was rapid and sustained. Moreover, Cbl was one of the major tyrosine-phosphorylated proteins in activated B cells. These results indicate that a relatively large fraction of Cbl is accessible to BCR-associated PTKs. The Src family kinases are thought to mediate the early phosphorylation events upon BCR triggering (7–9, 22). Consistent with Cbl being a substrate for Src kinases, a Fyn-Cbl complex was detectable prior to activation and increased further upon activation. In fact, Cbl represented the major Fyn-associated phosphotyrosyl polypeptide in activated Ramos cells. Smaller amounts of Cbl were also detected in association with Blk, Lck, and Lyn. However, the fraction of Cbl associated with Src family kinases was substantially smaller than the total tyrosine phosphorylated pool of Cbl (Fig. 4), suggesting that either Cbl is transiently recruited to Src family kinases and quickly released, or that additional cytoplasmic PTKs are involved. Since the SH3 and SH2 domains of Src family kinases can concurrently bind to phosphorylated Cbl and thus provide a high affinity interaction (23, 24), the second possibility is more likely. Consistent with this idea, a substantial fraction of Cbl became physically associated with Syk following B cell activation. Thus, Syk may represent one of the additional PTKs that phosphorylate Cbl. By analogy, a similar role of Syk/ZAP-70 in Cbl phosphorylation can be postulated in T cells particularly since a large non-Fyn/Lck-associated pool of phosphorylated Cbl is also found in activated T cells (23, 25). The possible involvement of other B cell PTKs, such as the Bruton’s tyrosine kinase (44, 45) remains to be investigated. Interestingly, Bruton’s tyrosine kinase SH3 domain can also bind to Cbl in vitro (46); however, this association has proven difficult to demonstrate in vivo.2

The differential association between Cbl and various Src family kinases was not due to inefficient immunoprecipitation of Lyn and Lck, as shown by immunoblotting with immunoprecipitating antibodies (data not shown), and by co-immunoprecipitation of several phosphotyrosyl polypeptides (Fig. 3). A lower association of Cbl with Lck compared to Fyn was also observed in our analyses of T cells (23, 25). The structural basis for differential interaction is not clear at present, but may include relative abundance of Src family proteins in cells, their relative affinities for Cbl versus other substrates, and the respective affinities of their SH3 and SH2 domains for Cbl. For example, a stronger in vitro binding to Cbl is observed with Fyn SH3 compared to Lck SH3 (23). Similarly, Lyn SH3 was able to interact with Cbl (36) whereas Blk SH3 was not (26). All analyzed Src family SH2 domains show some binding to tyrosine-phosphorylated Cbl in vitro, although this has not been carefully quantified (23, 25, 26). Regardless of the mechanism, however, differential association of Cbl with members of the Src family is consistent with distinct signaling roles of these kinases in B cell activation (47, 48).

Association of a large fraction of cellular Cbl with Grb2 before anti-IgM-stimulation of B cells (Fig. 4) is consistent with our observation in T cells where Grb2-Cbl interaction was

2 S. Pillai, personal communication.
Cbl Participates in B Cell Receptor Signaling

A large fraction of PI 3-kinase activity is associated with Cbl in anti-IgM-stimulated Ramos B cells. Immunoprecipitations carried out with the indicated antibodies (I. P. Abs., shown on top) from lysates of $5 \times 10^7$ unstimulated (–) or anti-IgM-stimulated (+) Ramos cell lines were subjected to lipid kinase assays using phosphatidylinositol (left panel) or phosphatidylinositol 4,5-bisphosphate (right panel) as substrate, as described under “Materials and Methods.” The reaction products were subjected to TLC to resolve phosphatidylinositol phosphates (PIP; left panel) or phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$; right panel) and visualized by autoradiography. Following the kinase reaction, bead-bound proteins were resolved by SDS-PAGE and immunoblotted with anti-PI 3-kinase p85 antibody (bottom). Right and left panels are separate experiments. All lanes within each panel are from a single exposure. The apparently unequal migration of PIP3 in the right panel reflects transposition of lanes from different parts of a TLC plate which ran with a slant.

In conclusion, we demonstrate that Cbl is a major substrate and co-immunoprecipitated a substantial level of PI 3-kinase activity. The activation dependence of these associations is consistent with our earlier observations that binding between PI 3-kinase p85 and Cbl in T cells was primarily mediated by PI 3-kinase p85 SH2 domains binding to py motifs on Cbl (25). Prior analyses have demonstrated that BCR activation induces tyrosine phosphorylation of CD19 creating a PI 3-kinase p85 SH2 domain-binding motif which allows PI 3-kinase recruitment (42). In addition, the SH3 domains of Src family kinases can interact with proline-rich sequences of PI 3-kinase p85 protein in vitro (47, 48), although the relative contribution of this mechanism to recruit PI 3-kinase to BCR in vivo remains to be clarified. In fact, relatively little PI 3-kinase activity co-immunoprecipitated with Fyn in T cells, whereas a large fraction of activity was associated with Cbl (25). Given the extent of Cbl-PI 3-kinase association in B cells, it is likely that this represents a major mechanism of activation-dependent recruitment of PI 3-kinase activity into phosphotyrosyl complexes, complementing the role of CD19 and Src family kinase SH3-mediated interactions with PI 3-kinase p85 (42, 47, 48).

In conclusion, we demonstrate that Cbl is a major substrate of tyrosine phosphorylation upon B cell antigen receptor activation, and represents a major in vivo associate for Grb2/SHC adaptors, the PI 3-kinase and the B cell PTKs Fyn and Syk. These findings strongly suggest the involvement of Cbl as a cytoplasmic signaling protein in tyrosine kinase-dependent activation of B lymphocytes through the B cell antigen receptor. Given the propensity of oncogenic Cbl to induce B cell leukemias (27), our results raise the possibility that Cbl may be involved in signaling cell proliferation and/or survival in B lymphocytes.

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