We and others have recently identified Cbl, the protein product of the c-cbl protooncogene, as an early tyrosine kinase substrate upon T cell activation and have shown that Cbl forms in vivo complexes with Src family tyrosine kinases, Grb2 adaptor protein, and the p85 subunit of PI-3 kinase. Here we show that Cbl associates with all three forms of the human Crk protein, predominantly CrkL, following T cell receptor activation of Jurkat T cells. Association between Cbl and Crk proteins was confirmed in normal human peripheral blood-derived T cells. In vitro, Cbl was able to interact with the Crk SH2 domain but not the SH3 domain. A phosphopeptide corresponding to a potential Crk SH2 domain-binding motif in Cbl (pYDVP) specifically inhibited binding between Cbl and Crk SH2 domain. Anti-Cbl antibody derived from T cells may be Cbl. Consistent with this possibility, the 4F4 antibody used to characterize the p116 polypeptide cross-reacted with Cbl protein when it was resolved on one- or two-dimensional gels. CrkL was constitutively associated with a substantial amount of the guanine nucleotide exchange protein C3G, and a fraction of the C3G protein was coimmunoprecipitated with Cbl in activated Jurkat T cells. These results suggest the possibility that Cbl may participate in a signaling pathway that regulates guanine nucleotide exchange on small G-proteins in T cells.

Tyrosine phosphorylation of intracellular substrates is an early and obligatory event in T cell receptor (TCR)-mediated lymphocyte activation. The TCR-CD3 components themselves lack intrinsic tyrosine kinase activity but physically associate with two Src family protein tyrosine kinases, p59Fyn (Fyn) and p56Csk (Lck) (1, 2). Fyn associates with the CD3 ζ/ε and TCR ζ/ε chains of the TCR-CD3 complex (3), while Lck associates with the CD4 and CD8 coreceptors (4, 5). Accumulating evidence suggests that these two kinases play critical roles in T cell development and activation (1, 2). A third cytoplasmic tyrosine kinase, ZAP-70, associates with the ζ and ε chains of the TCR-CD3 complex following their tyrosine phosphorylation and is required for propagating signals downstream of the Src kinases (6–8). All subsequent events, including phosphatidylinositol metabolism, mobilization of intracellular calcium, Ras activation, and eventual transcriptional modulation and mitogenesis are dependent upon initial tyrosine kinase activity (1, 2). Identification and functional characterization of tyrosine kinase substrates in activated T cells are therefore critical to our understanding of TCR signaling.

Recently, we described a Fyn/Lck SH3 domain-binding protein, p112, that served as one of the earliest tyrosine kinase substrates upon TCR stimulation (9). Subsequently, we and others have identified this protein as p120Cbl, the product of the c-cbl protooncogene (10, 11). Cbl was initially identified as the cellular homolog of the Cos N-S1 murine leukemia retroviral oncogene v-cbl, which induces pre-B and myeloid leukemias in infected mice and acutely transforms NIH 3T3 fibroblasts (12, 13). Another mutant form of Cbl with a 17-amino acid internal deletion has been isolated from a murine pre-B lymphoma 70/23 and is also transforming (13, 14). However, the mechanisms of oncogenic transformation, as well as the physiological role(s) of Cbl in signaling pathways are unknown. Therefore, identification of Cbl as an intermediate in a tyrosine kinase-dependent signaling pathway provided an impetus to elucidate its biochemical function. Moreover, Cbl tyrosine phosphorylation has also been demonstrated upon stimulation through the Fcy receptor (15), the B cell antigen receptor (16, 17), and the granulocyte-macrophage colony-stimulating factor and erythropoietin receptors (18), suggesting that Cbl plays a role in multiple antigen receptor- and mitogen receptor-associated tyrosine kinase activation pathways in hematopoietic cells.

The primary structure of Cbl reveals a lack of any obvious catalytic domains. However, Cbl possesses multiple potential tyrosine phosphorylation sites and proline-rich motifs, which could mediate concurrent association with SH2 and SH3 domain-containing polypeptides, respectively (19–21). Cbl also has a carboxyl-terminal leucine zipper, a motif known to promote homo- and heterodimerization of other proteins (22). Thus, Cbl is well-suited for a potential role in assembling...
intracellular signaling complexes. In support of this model, we have previously demonstrated the in vivo association of Cbl with two adaptor proteins, Grb2 and the p85 subunit of PI 3-kinase; Grb2 association was exclusively mediated by SH3 domains, while p85 associated with Cbl primarily through its SH2 domains (11). Another adaptor protein, Nck, has also been shown to bind to Cbl via SH3 domains (23).

Here we show that among a panel of isolated SH2 domains, that of Crk displays the highest binding to Cbl. This binding was specifically abrogated by a Cbl-derived phosphoryseryl peptide incorporating a consensus Crk SH2-binding motif. In vivo, all three cellular forms of Crk (CrkI, CrkII, and CrkL) bound to Cbl following TCR stimulation. We also show that the recently described p130SH2-related Crk-associated p16 of T cells (24) is Cbl. Finally, we show that C3G, the guanine nucleotide-releasing factor for a small G-protein Rap1 (25–27), was constitutively associated with Crk proteins and became associated with Cbl upon T cell activation. These results suggest a potential role of Cbl in connecting Src-family tyrosine kinase activation pathways with guanine nucleotide exchange on small G-proteins.

EXPERIMENTAL PROCEDURES

Peptides—The following phosphoryseryl peptides and their unphosphorylated analogs were synthesized and HPLC-purified, as described (28): the pT1MM motif peptide corresponding to CD28 Tyr256 (HSD-pTYMNTFLP; belface is SH2-binding motif) (19, 29); Cbl Tyr774 peptide (EDDGpYDVPP; human Cbl amino acids 770–781) (13). Cells—A subclone of the human T cell leukemia line Jurkat, J urkat JC MC (derived in our laboratory, and referred to herein as J urkat), was maintained in RPMI 1640 medium supplemented with 20 mM HEPES, pH 7.2–7.3, 2 mM l-glutamine, 1 mM sodium pyruvate, and 1% each of nonessential amino acids and penicillin/streptomycin (all from Life Technologies, Inc.), and 7% fetal calf serum (Hyclone). The JMC-HA-Cbl cell line was derived by retrovirus-mediated transfection of the J urkat JC MC cell line with hemagglutinin (HA) epitope-tagged human Cbl cDNA in pJ ZenNeo vector (14), as described (11). Transfectants were selected in G418 (Life Technologies, Inc.) and cloned by limiting dilution, and a high HA-Cbl-expressing clone was identified by immunoprecipitation with anti-HA tag antibody followed by anti-Cbl immunoblotting (data not shown). Normal peripheral T cells were derived by stimulating human buffy coat-derived mononuclear cells with phytohemagglutinin (Pharmacia Biotech Inc.; 1:2,000 dilution) followed by growth in recombinant IL-2 for 10 days. Cells were deprived of IL-2 for 1 day prior to activation.

Antibodies—The monoclonal antibodies used in this work were as follows: IgG1 (anti-Tyr; IgG1a) (30); SPV-T3b (anti-CD3; IgG2a) (31); 2A2 (anti-CD3; IgM; a gift from Ellis Reinherz, Dana-Farber Cancer Institute, Boston); anti-Crk (IgG2a) (S12620, Transduction Laboratories); anti-CrkII (sc-289), anti-CrkL (sc-319), and anti-Grb2 (sc-255) (from Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-Cbl (Santa Cruz Biotechnology sc-170, or generated in our laboratory by immunizing with an identical C-terminal 15-amino acid peptide); and anti-C3G (a gift from Dr. Michiyuki Matsuda, Nihon, Tokyo, Japan). For immunoprecipitation with 4F4 antibody, rabbit anti-mouse Ig (Zymed) was used for coupling to protein A-Sepharose. Horseradish peroxidase-conjugated goat antibody (Cappel-Organon Technika, Durham, NC), donkey anti-rabbit Ig, or sheep anti-mouse Ig (both from Amersham Corp.) were used as second step reagents for Western blotting.

Glutathione S-transferase (GST) Fusion Proteins—The Fyn and Lck GST fusion protein constructs in pGEX2T K and p85 SH2(N+C) in pGEX2T have been previously described (9, 11, 32). Abl and V-Crk SH2 or SH3 domains and the N-terminal SH2 domain (SH2-N) of GTPase-activating protein in pGEX2T were provided by Bruce Mayer (Childrens Hospital, Boston) (33–35). GST fusion proteins were generated and purified as described previously (9, 11, 32).

Activation of T Cells—Cells (5 × 10^5/ml) were incubated at 37°C in RPMI 1640, 20 mM HEPES either in the absence (negative control) or presence of anti-CD3 monoclonal antibody SPV-T3b for 2 min, lysed at 1.25–2.5 × 10^6/ml in cold lysis buffer (0.5% Triton X-100 (Fuka), 50 mM Tris, pH 7.5, 150 mM sodium chloride, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and pepstatin, 1 mM sodium orthovanadate, and 10 mM sodium fluoride), and clarified by centrifugation. Normal IL-2-expanded T cells were grown in IL-2-free medium for 1 day followed by stimulation with an IgM anti-CD3 antibody (2A2), as for Jurkat T cells.

Immunoprecipitations and GST Binding Reactions—Optimal amounts of antibodies were added to cell lysates (2.5–5 × 10^7 cell equivalents) precladded with NRS and Staphylococcus aureus Cowan I strain (Pansorbin, Calbiochem). Incubations were performed for 1–4 h at 4°C, at which time 20 μl of protein A-Sepharose CL-4B beads (Pharmacia) was added. Cbl incubation was continued for an additional 45–60 min. The beads were then washed 6 times in cold lysis buffer, and immunoprecipitated proteins were eluted by boiling in sample buffer under reducing conditions.

For immunodepletion studies, cell lysates were incubated with two serial aliquots of antibodies together with protein A-Sepharose beads, and the resulting precladed supernatant was used for further immunoprecipitations.

For GST fusion protein binding reactions, 5–10 μg of fusion protein noncovalently immobilized on glutathione-Sepharose beads was incubated for 1 h with cell lysate and washed 6 times with lysis buffer, and bound polypeptides were eluted in sample buffer. For peptide competition, both lysate and fusion protein were preincubated with peptides for 30 min prior to the binding assay. For immunoblots, SDS-PAGE, Isoelectric Focusing, and Immunoblotting—Samples were resolved by SDS-PAGE (7.5–10.5% gels) under reducing conditions and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA). Immunoblotting was carried out as described (11), using horseradish peroxidase-conjugated second step reagents and enhanced chemiluminescence (ECL; reagents from Dupont NEN). Reprobing of filters was performed as described (11).

For two-dimensional gel analysis, proteins were resolved by isoelectric focusing (IEF) in pH 3.5–10.0 ampholines (Pharmacia) in the first dimension, followed by SDS-PAGE and Western blotting, as described previously (11).

RESULTS

Cbl SH2 Domain Shows Preferential Binding to Cbl—We and others have previously identified Cbl, the protein product of the c-cbl protooncogene, as an early substrate of tyrosine kinases following T cell receptor ligation (10, 11). Tyrosine-phosphorylated Cbl forms SH2 domain-mediated complexes with Src family tyrosine kinases and the p85 subunit of PI 3-kinase (11). Because Cbl contains numerous potential tyrosine phosphorylation sites, we assessed additional SH2 domains for binding to Cbl. For this purpose, GST fusion protein binding reactions were carried out with detergent lysates of resting and activated Jurkat cells. Bound Cbl was detected by anti-dbl and anti-phosphotyrosine (Tyr(P)) immunoblotting.

GST fusion proteins of Fyn (Fig. 1A, upper panel, lanes 5 and 6) and Lck SH2 domains (lanes 7 and 8), but not the GST alone (lanes 3 and 4) bound to Cbl, primarily in lysates of anti-CD3-stimulated Jurkat cells, as previously reported (11). Cbl binding to Abl SH2 (lanes 9 and 10) was comparable with that of Fyn and Lck SH2 but was barely detectable in GTPase-activating protein SH2-N binding reactions (lanes 11 and 12). Notably, the strongest binding to Cbl was seen with Crk SH2 (lanes 13 and 14); substantial binding was observed in lysates of unstimulated cells and increased further upon T cell activation. A 20-fold shorter exposure of this blot emphasizes the markedly higher binding of Cbl to Crk SH2 (Fig. 1A, middle panel). Equal Cbl signals were present in lysates of unstimulated and stimulated cells (upper panel, lanes 1 and 2), indicating that the SH2 domain binding to Cbl was activation-dependent. Anti-Tyr(P) reprobing of the blot showed activation-induced tyrosine phosphorylation of Cbl, which correlated with its capacity to bind to various SH2 domains (Fig. 1A, lower panel).

As noted above, a substantial amount of Cbl bound to Crk SH2 even in lysates of unstimulated cells, where overall tyrosine phosphorylation was relatively low. In order to establish
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that Cbl-Crk SH2 interaction was mediated by phosphoryseryl peptide binding, we carried out competition experiments with phosphoryseryl peptides. Cbl amino acids 774–777 (YDVP) associate with Crk SH2 domain and specific inhibition of this binding by a Cbl-derived phosphotyrosyl peptide containing a consensus Crk SH2-binding motif, we investigated whether Cbl binding to Crk SH2 domain and selective inhibition of binding by Cbl-amino acids 774–777 (YDVP) (13) corresponds to a consensus high affinity phosphotyrosyl motif. A, binding of Cbl to Crk SH2 domain compared to other SH2 domains. Binding reactions were carried out by incubating lysate from 2.5 × 10^6 unstimulated (−) or anti-CD3 (SPV-T3b)-stimulated (+) Jurkat cells (in a 2-ml volume) with 10 μg of the indicated GST fusion proteins noncovalently immobilized on glutathione-Sepharose beads (5 μl of packed beads) for 1 h. Whole cell lysate (106 cells) or binding reactions were subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes, and blotted with anti-Cbl (upper and middle panels) or anti-Tyr(P) antibody (lower panel), followed by horseradish peroxidase-conjugated second step antibodies and detection by ECL. The middle panel represents a 20-fold lower exposure of the membrane shown in the top panel, emphasizing the marked higher binding of Cbl to Crk SH2 as compared with other SH2 domains. The lower panel represents anti-Tyr(P) reprobing of the same membrane shown in the upper panels. B, specific competition of Cbl binding to GST-Crk SH2 domain by Cbl Tyr(P)774 peptide. Competing peptides were separately added to bead-bound fusion proteins and cell lysate at the indicated concentrations (shown in μM; 0, no peptide). After 30 min, beads and lysate were mixed, and binding reactions and immunoblotting with anti-Cbl antibody were carried out as in Fig. 1A. Peptides were: EDGYDVPKPPV (pYDVP; Cbl amino acids 770–881); HSDYMTPR (pYMMPN; p85 SH2-specific); HSDYMTPR (YMMPN), C, lack of Cbl binding to Crk SH3 domain. Binding reactions with the indicated GST fusion proteins and anti-Cbl immunoblotting were carried out as in Fig. 1A.

In contrast to prominent binding of Cbl to Crk SH2 domain, no Cbl binding to Crk SH3 domain was observed under conditions that revealed a substantial binding of Cbl to Fyn SH3 fusion protein (Fig. 1C). These results are consistent with our earlier studies, which failed to detect a 120-kDa phosphotyrosyl protein in Crk SH3-binding reactions from T cells (9).

TCR Activation-dependent Association of Crk Proteins with Cbl—In view of the preferential in vitro binding of Cbl to the Crk SH2 domain and specific inhibition of this binding by a Cbl-derived phosphotyrosyl peptide containing a consensus Crk SH2-binding motif, we investigated whether Cbl associates with Crk proteins in vivo. Three forms of Crk have been identified in mammalian cells. CrkI and CrkL, protein products of two related but distinct genes, each contain an SH2 domain and two SH3 domains (34, 37, 38). CrkI, an alternatively spliced form of CrkII, also has an SH2 domain, but only one SH3 domain (37). To assess Cbl-Crk interactions in vivo, lysates of unstimulated or anti-CD3-stimulated (−) Jurkat T cells were immunoprecipitated with anti-Cbl or anti-Crk antibodies and analyzed by anti-Tyr(P) immunoblotting (Fig. 2).

Some basal tyrosine phosphorylation was observed on directly immunoprecipitated Cbl, with a substantial increase in Tyr(P) signal upon T cell activation (Fig. 2, upper panel, lanes 3 and 4). While little phosphotyrosine signal was detected in immunoprecipitates of unstimulated Jurkat cells carried out with anti-Crk (which immunoprecipitates both CrkI and CrkII) (lane 5) or anti-CrkI antibody (lane 7), a prominent 120-kDa phosphotyrosyl polypeptide was observed with each of these antibodies following anti-CD3-stimulation (lanes 6 and 8). A low but easily detectable p120 signal was observed in anti-Tyr(P) immunoblotting of anti-CrkL immunoprecipitate from unstimulated cells with a marked increase in signals upon activation of cells (lanes 9 and 10). Among the three antibodies used, that against CrkL showed the highest p120 signals. In each case, Crk-associated p120 comigrated with Cbl. Minor unidentified tyrosine-phosphorylated species of 100, 75, and 36–40 kDa were also observed in anti-CrkL immunoprecipitates of activated T cells, particularly upon longer exposures (lane 10 and data not shown).

To assess if the Crk-associated tyrosine-phosphorylated p120...
was Cbl, we reprobed the blot with an anti-Cbl antibody (Fig. 2, lower panel). This analysis demonstrated that each of the three antibodies against Crk proteins co-immunoprecipitated Cbl. Cbl coimmunoprecipitation with anti-Crk and anti-CrkII antibodies was observed in lysates of anti-CD3-stimulated but not unstimulated Jurkat cells (compare lanes 5 and 7 with lanes 6 and 8). Small amounts of Cbl were observed in anti-CrkL immunoprecipitates prior to activation and increased substantially upon anti-CD3 stimulation of cells, correlating with the levels of Cbl tyrosine phosphorylation seen by anti-Tyr(P) immunoblotting (lanes 9 and 10). Overlay of anti-Tyr(P) and anti-Cbl blots showed that these bands coincided exactly (not shown).

While the above results clearly demonstrated Cbl association with CrkII and CrkL, we could not directly assess this for CrkI. To address this question, cell lysates were first precleared with either a control antibody (NRS) or with anti-CrkII antibody followed by immunoprecipitation with anti-Crk (CrkI- and CrkII-reactive) or anti-CrkII antibody. Anti-Crk immunoblotting (Fig. 3A, upper panel) revealed that only a small amount of CrkII could be immunoprecipitated by anti-Crk (compare lanes 1 and 2 with lanes 5 and 6) or anti-CrkII antibody (compare lanes 3 and 4 with lanes 7 and 8) in anti-CrkII-immunodepleted lysates compared with control (NRS-precleared) lysates. Furthermore, anti-Crk antibody immunoprecipitated equal amounts of CrkI protein in control and anti-CrkII-precleared lysates (compare lanes 1 and 2 with lanes 5 and 6). Anti-Tyr(P) immunoblotting (Fig. 3A, upper panel) demonstrated that CrkII preclearing completely removed the p120 band in anti-CrkII immunoprecipitates (compare lanes 4 and 8), whereas p120 was still observed in anti-Crk antibody immunoprecipitate (compare lanes 2 and 6). Anti-Cbl immunoblotting confirmed that p120 was Cbl (not shown). These results show that CrkI protein also associates with Cbl, thereby strongly indicating that all three forms of Crk associate with Cbl in activated T cells.

To assess if Cbl was the only 120-kDa Tyr(P) polypeptide associated with Crk proteins, we immunodepleted Cbl from cell lysates and then carried out anti-CrkL immunoprecipitations. Anti-Cbl immunoprecipitation followed by anti-Tyr(P) and anti-Cbl immunoblotting (Fig. 3B) demonstrated a marked decrease (although not a complete loss) of Cbl protein in anti-Cbl-immunodepleted lysates (compare lanes 1 and 2 with lanes 5 and 6). Significantly, anti-CrkL immunoprecipitation from Cbl-depleted lysates showed undetectable levels of Cbl and, concurrently, an essentially complete loss of the p120 band in anti-Tyr(P) blot (compare lanes 4 and 8). These results indicate that Cbl is the major, and perhaps the only, Crk-associated 120-kDa Tyr(P) polypeptide in T cells.

Association of Cbl with Crk Proteins in Normal Human T Cells —To exclude the possibility that Cbl-Crk interaction may be a peculiarity of the leukemic cell line Jurkat, we assessed Cbl-Crk association in normal human T cells. Short-term T cell cultures established by phytohemagglutinin stimulation following growth in IL-2, were stimulated with anti-CD3 antibody 1 day after IL-2 withdrawal to reduce basal tyrosine phosphorylation.

Anti-Tyr(P) immunoblotting of anti-Cbl immunoprecipitates showed a prominent activation-dependent tyrosine phosphorylation of Cbl (Fig. 4, upper panel, lanes 1 and 2). Anti-Cbl immunoblotting revealed equal amounts of Cbl in both lanes (lower panel). Significantly, a 120 Tyr(P) polypeptide comigrating with Cbl was observed in anti-Crk immunoprecipitate from activated cells (lane 6). Easily detectable 120-kDa Tyr(P) polypeptide signal was observed in anti-CrkL immunoprecipitate from unstimulated cells but increased markedly upon activation (upper panel, lanes 7 and 8). Anti-Cbl blotting demonstrated the p120 Tyr(P) polypeptide to be Cbl. Thus, Cbl shows an activation-dependent association with Crk proteins in normal T cells. Interestingly, anti-Crk and anti-CrkL antibodies also showed prominent activation-dependent co-immunoprecipitation of unidentified 75–80-kDa (upper panel) and 36–40-kDa (not shown) Tyr(P) polypeptides, similar in size to minor bands seen in anti-CrkL immunoprecipitations from activated Jurkat T cells.

The T Lymphocyte p130Cas-related p116 Is Cbl —In the course of our studies, it was reported that Crk protein associates with
a 116-kDa Tyr(P) protein, p116, in activated T lymphocytes (24). These workers noted that p116 was reactive with the monoclonal antibody 4F4, previously shown to be directed against Src substrate p130Cas (39, 40). In view of the essentially complete immunodepletion of CrkL-associated p120 by anti-Cbl antibody, we investigated whether p130Cas-related Crk-associated p116 is Cbl or a distinct polypeptide comigrating with Cbl. For this purpose, we compared 4F4 and anti-Cbl immunoblots of various immunoprecipitations from unstimulated and anti-CD3-stimulated Jurkat lysates.

The 4F4 antibody immunoblotted with a 120-kDa protein in anti-CrkL (Fig. 5A, upper panel, lanes 3 and 4), anti-Tyr(P) (lanes 7 and 8), and 4F4 (lanes 9 and 10) immunoprecipitates of anti-CD3-stimulated Jurkat cell lysates with little or no reactivity in immunoprecipitates from unstimulated cells, as expected. Surprisingly, the 4F4 antibody prominently immunoblotted with the directly immunoprecipitated Cbl protein from activated Jurkat cells with only low reactivity to that from unstimulated cells (lanes 5 and 6). Additionally, the 4F4 antibody specifically reacted with a number of other proteins in anti-Tyr(P) immunoprecipitates (lane 8), some of which were also detected in 4F4 immunoprecipitates (lane 10), suggesting that the 4F4 epitope is induced on a number of proteins following T cell activation. This result is consistent with an earlier observation that 4F4 antibody reacts with several phosphotyrosyl proteins and that this reactivity is inhibited by phosphotyrosine (41).

Reprobing of the filter with anti-Cbl antibody (Fig. 5A, lower panel) showed equivalent amounts of Cbl in anti-Cbl immunoprecipitations from unstimulated and activated lysates (compare lanes 5 and 6), suggesting that the 4F4-reactive epitope was induced on Cbl upon T cell activation. The reactivity of the 4F4 antibody with the 120-kDa species in anti-CrkL (lanes 3 and 4) and anti-Tyr(P) immunoprecipitates (lanes 7 and 8) correlated with their coimmunoprecipitation of Cbl. Low levels of Cbl were detectable by anti-Cbl immunoblotting in 4F4 immunoprecipitates from activated Jurkat cells (compare lanes 9 and 10, lower panel); although the amount of coimmunoprecipitated Cbl was relatively small, it was specific, as no Cbl was detected in control lanes (lanes 1 and 2).

Separately, we reprobed the filter shown in Fig. 3B with the 4F4 antibody (Fig. 5B). This analysis showed that immunodepletion with anti-Cbl eliminated the 4F4-reactive p120 band in anti-CrkL immunoprecipitates.

To further confirm that 4F4 antibody reacts with Cbl, we subjected anti-Cbl immunoprecipitates from activated Jurkat cell lysates to two-dimensional gel analysis (IEF, followed by...
RESULTS presented above strongly suggest that p130Cas showed that the 4F4-reactive spots aligned exactly with near the origin of the IEF gel. Anti-Cbl reprobing of the filter spotsofapproximately120kDaandathirdspottosimilarsize Cbl). The 4F4 antibody recognized two distinct antibodies (Fig. 5). SDS-PAGE), and serial immunoblotting with 4F4 and anti-Cbl antibodies (Fig. 5C). The 4F4 antibody recognized two distinct spots of approximately 120 kDa and a third spot of similar size near the origin of the 1E6 gel. Anti-Cbl reprobing of the filter showed that the 4F4-reactive spots aligned exactly with spots A, C, and D identified in the anti-Cbl blot. Collectively, the results presented above strongly suggest that p130Cas-related Crk-associated p116 (24) is Cbl.

Activation-induced Association of the Guanine Nucleotide Exchange Protein C3G with Cbl—Recent studies have revealed a role for Crk proteins in regulating Ras activation that requires both Crk SH2 and SH3 domains (26). It has now been shown that the Crk SH3 domain directly binds to two guanine nucleotide exchange proteins, SOS and C3G, which regulate Ras and Rap1, respectively (25–27, 42, 43). Thus, Crk has been suggested to play a role similar to Grb2 by binding to phosphotyrosyl proteins via its SH2 domain and thereby recruiting SH3-bound guanine nucleotide exchange proteins into tyrosine kinase signaling machinery. Since Cbl interacts with the SH2 domain of Crk proteins, we wished to investigate whether this would allow complex formation between Cbl and SOS or C3G.

First, we examined whether SOS and/or C3G polypeptides were associated with CrkL protein. Lysates of unstimulated and anti-C3D-stimulated Jurkat cells were immunoprecipitated with various antibodies and immunoblotted with anti-SOS or anti-C3G antibody (Fig. 6A). A small but readily detectable pool of SOS was observed in association with Grb2 in unstimulated lysates (lower panel, lane 5); a substantial increase in this association was observed upon anti-C3D activation (lane 6), confirming previous results (11, 44). In contrast, SOS was not detectable in anti-CrkL immunoprecipitates (lanes 7 and 8), except after prolonged exposure of the blots (not shown). A relatively large fraction of C3G was constitutively associated with CrkL, and this association remained unchanged upon activation (Fig. 6A, upper panel, lanes 7 and 8). Notably, C3G was not detected in anti-Grb2 immunoprecipitates (lanes 5 and 6) except after overexposure of the blot (data not shown). These analyses indicated that C3G is preferentially associated with CrkL as compared with Grb2 in T cells and suggested that the C3G-CrkL complex may associate with Cbl upon T cell activation.

In initial experiments using Jurkat cells, a small amount of C3G could be observed to associate with Cbl but was detectable only after long exposures of gels (data not shown). To facilitate the analysis of this complex, we derived a transfectant of the Jurkat-J MC cell line, J-MC-HA-Cbl (see “Experimental Procedures”), which expresses about 5-fold higher levels of Cbl protein as compared with parental cells (data not shown). Similar to parental Jurkat cells (Fig. 2), anti-CD3 stimulation of J-MC-HA-Cbl induced tyrosine phosphorylation of a number of cellular polypeptides including the transfected Cbl (data not shown), and CrkL immunoprecipitates from these cells showed a substantial level of constitutively associated C3G (Fig. 6B, lanes 7 and 8). Significantly, a small amount of C3G was specifically observed in anti-Cbl immunoprecipitates from activated cells (lane 6). These results indicate that a Cbl-CrkL-C3G ternary complex is induced by T cell activation.

DISCUSSION

In contrast to the receptor tyrosine kinases, whose autophosphorylation creates multiple distinct phosphopeptide motifs as docking sites for SH2 domain-containing signaling proteins (19, 21, 29, 45), the tyrosine phosphorylation motifs associated with the lymphocyte antigen receptors lack significant diversity (1, 2), and are therefore unlikely to directly recruit multiple signaling proteins. Identification and characterization of polypeptides that fulfill such a role is likely to enhance our understanding of lymphocyte antigen receptor signal transduction.

Recently, we identified one such substrate, p120, which interacts with Fyn and Lck SH3 domains via proline-rich peptide motifs and with Fyn and Lck SH2 domains via phosphotyrosyl interactions (9). We and others have identified p120 as Cbl, the product of the c-cbl protooncogene, and have shown it to associate with PI 3-kinase by binding to SH2 domains of the p85 subunit and with Grb2 by binding to its SH3 domains (10, 11, 46). The SH2/SH3 domain-containing adaptor protein Nck has also been shown to associate with Cbl via its SH3 domains (23), although we have been unable to detect this association in T lymphocytes (data not shown). Together, these observations are consistent with a potential role of Cbl in assembling multiple T cell signaling proteins into complexes, as suggested by the presence of an extended proline-rich region and multiple potential tyrosine phosphorylation sites throughout Cbl (13).

Here we report the association of tyrosine-phosphorylated Cbl with the Crk family of SH2/SH3 domain adaptor proteins in activated T lymphocytes. Such an interaction was suggested by a remarkably strong in vitro binding of v-Crk SH2 domain to Cbl and the failure of Crk SH3 domain to bind to this polypeptide. Cbl amino acid residues 774–777 (YDVP) represented a potential Crk SH2 domain-binding motif since Crk SH2 was shown to select a pYDVP motif from degenerate phosphopeptide libraries (36). Indeed, a Tyr(P) peptide incorporating the Cbl YDVP motif specifically abrogated Crk SH2-Cbl binding, while it had no effect on PI 3-kinase p85-Cbl binding (Fig. 1B). In vivo, Cbl became associated with all three Crk isoforms (CrkI, CrkII, and CrkL) in an activation-dependent manner; however, association with CrkL was most prominent (Figs. 2 and 3). Cbl tyrosine phosphorylation and its prominent association with Crk proteins, in particular CrkL, were also observed upon TCR stimulation of normal peripheral blood-derived T
lymphocytes (Fig. 4), indicating that this association is not a peculiarity of transformed cells such as J urkat. Whether preferential association of Cbl with CrkL reflects a higher abundance of CrkL protein in T cells, a higher affinity of CrkL SH2 domain for Cbl or the possible influence of surrounding non-SH2 sequences of CrkL remains unknown at present. Notably, the affinity of Crk SH2 for phosphorysyl p130cas was shown to be increased by the N-terminal 32 residues of the v-Crk protein (47).

Our analyses show that Cbl is the major, and most likely the only 120-kDa Tyr(P) polypeptide associated with Crk proteins. This was supported by comigration of Crk-associated p120 with Cbl, its immunoreactivity with anti-Cbl antibodies, and an essentially complete immunodepletion of this band by preincubation with anti-Cbl antibody. Furthermore, Cbl was the major Crk-associated Tyr(P) polypeptide in J urkat T cells, although minor 100–, 75–80-, and 36–40-kDa Tyr(P) polypeptides were also observed upon longer exposures, particularly with anti-CrkL antibody (Figs. 2 and 3 and data not shown). Interestingly, analysis of normal T cells revealed that 75–80-kDa (Fig. 4) and 36–40-kDa Tyr(P) polypeptides (data not shown) were prominently associated with Crk proteins. Although the identity of these polypeptides is unknown, these results suggest that Crk adaptor proteins may be involved in multiple signaling pathways in primary T cells.

In view of our findings that Cbl interacts with Crk proteins in activated T cells and a complete immunodepletion of the Crk-associated 120-kDa Tyr(P) polypeptide by anti-Cbl antibody, we were intrigued by a recent report (24) of a Crk-associated 116-kDa Tyr(P) polypeptide in activated T cells. This protein was shown to cross-react with an antibody, 4F4, raised against the Src substrate p130cas (39) and recently identified as Crk-associated p130cas (40). The primary structure of Cbl does not show any significant homology with p130cas, suggesting that the 4F4-reactive, putative p130cas-related T cell polypeptide may be tyrosine-phosphorylated Cbl. Consistent with this possibility, another study had shown that 4F4-reactivity of a Fyn-associated T cell p116 polypeptide was sensitive to phosphotyrosine competition (41). Immunoblotting and removal of the Crk-associated 4F4-reactive p120 band by anti-Cbl antibody, together with the reactivity of 4F4 antibody with Cbl spots resolved on two-dimensional gels, demonstrated that p130cas-related p116 and Cbl are identical. Notably, however, only a small quantity of Cbl was observed in 4F4 immunoprecipitates (Fig. 5A). This likely represents an inefficient immunoprecipitation by this IgM subclass antibody and/or its selective immunoprecipitation of only tyrosine-phosphorylated Cbl. Indeed, 4F4 immunoblot showed stronger p120 signals in anti-Cbl compared with 4F4 immunoprecipitations (Fig. 5A). Consistent with the previous suggestion that 4F4 antibody recognizes selected phosphorysyl peptide motifs (41), this antibody specifically blotted with a number of additional T cell proteins immunoprecipitated by anti-Tyr(P) antibody (Fig. 5A).

In addition to its rapid tyrosine phosphorylation following TCR ligation, Cbl is a major target of tyrosine kinases coupled to the B cell antigen receptor (16, 17), granulocyte-macrophage colony-stimulating factor, and EPO receptors on hematopoietic cells (18) and the Fcγ receptor on myelomonocytic cells (15). It will be of interest to know whether Crk is a major Cbl-associating protein in these other signaling pathways as well. In this regard, both CrkL and Cbl are major tyrosine-phosphorylated substrates of BCR/Abi in Philadelphia-positive leukemic cells, and CrkL-Cbl complexes have been demonstrated in these cells (14, 48–50).

The prominent association of Cbl with Crk proteins suggested the possibility that Crk-mediated interactions may be involved in Cbl function. Previous studies have demonstrated that Crk interacts via its N-terminal SH3 domain with the guanine nucleotide exchange factors SOS and C3G (24–26, 42, 43), suggesting that Crk proteins activate Ras and/or other small G-protein signaling pathways. This possibility was consistent with the requirement of the SH3 domain for the transforming ability of V-Crk (34). Indeed, overexpression of Crk was shown to induce Ras-dependent differentiation of PC12 neuronal cells; functional SH2 and SH3 domains were required for this effect (42). Recent experiments have shown that the preferred target for C3G is Rap1 rather than Ras, suggesting that Crk proteins may link activated receptors to multiple small G-proteins. We observed a strong constitutive association of CrkL with the guanine nucleotide exchange factor C3G in J urkat T cells (Fig. 6). Importantly, a fraction of C3G was co-immunoprecipitated with Cbl in activated cells. Thus, Cbl, through its interaction with Crk proteins, becomes coupled to a guanine nucleotide exchange factor. These findings strongly suggest the possibility that tyrosine phosphorylation of Cbl may provide one mechanism to link upstream tyrosine kinases to small G-protein regulation in T cells.

Grb2 showed an easily detectable association with SOS and this association increased further upon T cell activation (Fig. 6A), as reported earlier (11, 44). In contrast, very little C3G was associated with Grb2 in T cells. Conversely, little SOS was associated with CrkL. Thus, in T cells, the Crk proteins and Grb2 appear to preferentially couple to distinct guanine nucleotide releasing factors that regulate Rap1 and Ras, respectively. Given that Cbl and SOS both bind to Grb2 SH3 domains and provide their alternate ligands (11, 46), Cbl-Crk-C3G complex formation may provide a mechanism for coupling tyrosine phosphorylation with small G-protein regulation in T cells, distinct from the previously examined Shc-Grb2-SOS and p36/38-Grb2-SOS complexes implicated in Ras regulation (44, 51–54). Consistent with this scheme, SOS was not detected in association with Cbl before or after T cell activation (11, 46).

In view of the results discussed above, the activation-dependent recruitment of the PI 3-kinase to tyrosine-phosphorylated Cbl (11, 46) is quite intriguing. PI 3-kinase is thought to play a role as either a regulator or effector of Ras function (55–57). Distinct Tyr(P) motifs favorable for binding to SH2 domains of PI 3-kinase p85 (e.g. Cbl residues 371–374 (YCEM) and 731–734 (YEAM)) and Crk (pYDVP; Cbl residues 774–777, Fig. 1B) (19, 36) are found in Cbl (13). Thus, Cbl might simultaneously recruit multiple modulators of small G-proteins in the vicinity of activated T cell receptors, perhaps as a signal amplification mechanism.

In conclusion, we demonstrate a T-cell activation-dependent SH2 domain-mediated association of Crk proteins, in particular CrkL, with tyrosine-phosphorylated Cbl, and show that activation induces a complex of Cbl with the guanine nucleotide exchange protein C3G. Together with the known oncogenicity of altered forms of Cbl as well as Crk, these findings suggest that Crk-Cbl complexes may play a role to mediate a growth-related signal downstream of the lymphocyte antigen receptors.

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REFERENCES

1. Weiss, A., and Littman, D. R. (1994) Cell 76, 263–274
2. Perlmutter, R. M., Levin, S. D., Appleby, M. W., Anderson, S. J., and Alberda-Ela, J. (1993) Annu. Rev. Immunol. 11, 483–499
3. Timson Gauen, L. K., Kong, A. N., Samelson, L. E., and Shaw, A. S. (1992) Mol.

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