The proto-oncogene product, Cbl, is a 120-kDa protein present in lymphocytes that contains numerous PXXP motifs in its COOH-terminal region and constitutively binds the SH3-containing adaptor protein Grb2. Cross-linking of CD3 and CD4 receptors in Jurkat T cells causes tyrosine phosphorylation of Cbl and its association with phosphatidylinositol 3-kinase (Meisner, H., Conway, B., Hartley, D., and Czech, M. P. (1995) Mol. Cell. Biol. 15, 3571–3578). Here we demonstrate that Cbl is stably associated with the adaptor protein Grb2, and that activation of T-lymphocytes by cross-linking of the T-cell antigen receptor recruits PI 3-kinase into Cbl complexes (6). These data suggest that Cbl may play an important role in facilitating signaling mechanisms in T-cells.

Although the possible role of Cbl in hematopoietic cell signaling has been emphasized previously (6, 7, 10), the presence of Cbl in HELa cells (9) prompted us to evaluate the role of this protein in other signaling systems. Of particular interest is the association of Cbl with Grb2, which is distributed widely among cell types. Grb2 is composed of one SH2 and two SH3 domains that link proteins containing phosphotyrosine to cellular proteins with proline-rich regions (11, 12). For example, Grb2 is known to bind via its SH2 domain to phosphotyrosine residues in the EGF receptor (13–15) and to insulin receptor substrate 1 (IRS-1), which serves as a docking protein for SH2 domains in insulin-sensitive cells (16). The NH2-terminal SH3 domain binds stably to Cbl in hematopoietic cells (6, 7), as well as to dynamin (17) and C3G (18) in other cell types. Grb2 also has a major role in the control of Ras signaling by binding to the guanine nucleotide-releasing factor Sos (19–21). Both Sos and Cbl possess the identical proline-rich sequence PPVPPPR that has been identified as the region in Sos that binds to the Grb2 SH3 domain (19). In this report we show that Cbl is indeed present in all cell types examined, and that addition of EGF to cultured human kidney cells causes the recruitment of Cbl into a complex containing the EGF receptor and its rapid tyrosine phosphorylation. Our data indicate that such association of Cbl with the EGF receptor may be mediated through Grb2.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The polyclonal Grb2 antibody was made by immunizing rabbits with a full-length GST-Grb2 fusion protein, obtained as a gift from Dr. J. Schlessinger. The sheep anti-human EGF receptor polyclonal antibody was a gift from Dr. Roger Davis. Polyclonal Cbl antibody was purchased from Santa Cruz Inc. The Grb2 mAb was from Transduction Laboratories, and anti-phosphotyrosine mAb 4G10 from UB1. Horseradish peroxidase-conjugated rabbit anti-sheep IgG, horseradish peroxidase conjugated rabbit anti-mouse IgG, and protein A-horseradish peroxidase were from Amersham. Detection of horseradish peroxidase was by chemiluminescence (Boehringer Mannheim). OKT3 and OKT4 mAbs were purified from supernatants of hybridoma cells by passage over protein G-Sepharose in a high salt buffer (22). Peptides corresponding to the proline-rich region of murine Sos (EVPPVPPVPVPPRRRE) (19, 23) or a nonrelevant sequence (ASGRQAGQGSDKTEPELFHPGADSOV) were synthesized in the Protein Synthesis Facility at the University of Massachusetts Medical Center and purified by reverse phase high performance liquid chromatography.

Cell Culture—The Jurkat cell line E6-1, obtained from the ATCC, Rockville, MD, was grown in RPMI 1640 supplemented with 7% fetal calf serum (Life Technologies, Inc.), 2 mM glutamine, 10 mM Hepes, pH 7.4, 50 μM 2-mercaptoethanol, and penicillin/streptomycin. The embryonic human kidney 293 cell line was passaged in Dulbecco’s minimal Eagle’s medium plus 10% calf serum.

Cell Stimulation and Preparation of Cell Lysates—Jurkat cells were serum starved for 4 h, resuspended in serum-free medium, and acti-
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RESULTS AND DISCUSSION

We previously identified a major tyrosine-phosphorylated 120-kDa protein in T-cell receptor-activated Jurkat T-cells as the product of the Cbl proto-oncogene (6). Although this protein has been found primarily in hematopoietic cells, Fig. 1 shows that Cbl is present in Nonidet P-40 lysates from several other cells and tissues. In addition to Jurkat cells, high levels were found in two kidney cell lines (COS and 293), while CHO cells and 3T3-L1 preadipocytes expressed approximately 10% of this level. In differentiated 3T3-L1 adipocytes or rat adipose tissue, the concentration of Cbl was about 5% that of Jurkat cells. The low expression of Cbl in adipose cells is in agreement with the sensitivity of these cells to EGF (19), led us to determine whether EGF receptor activation results in tyrosine phosphorylation of this protein. Fig. 2 (lanes 1–5) reveals that stimulation of 293 cells with EGF for 2 min led to the appearance of a tyrosine-phosphorylated 120-kDa protein in total lysates (lane 2) and in anti-Cbl immunoprecipitates (lane 5) that blotted with Cbl antiserum (lower panel). EGF action also resulted in the appearance of other phosphotyrosine bands in anti-Cbl precipitates, most noticeably at 170 kDa. In Jurkat T-lymphocytes, cross-linking of the CD3/CD4 receptors (Fig. 2, lanes 6–10) increased tyrosine phosphorylation of Cbl and led to the co-precipitation of bands at 100, 83, 72, and 60 kDa, but not at 170 kDa, as detected with 4G10 antibody. As depicted in the lower panel of Fig. 2, the amount of Cbl that was immunoprecipitated with anti-Cbl antibody is similar in stimulated versus unstimulated 293 or Jurkat cells. These data demonstrate that EGF action causes marked tyrosine phosphorylation of Cbl in 293 cells. Moreover, EGF also enhances either the association of Cbl with tyrosine-phosphorylated proteins or the tyrosine phosphorylation of proteins constitutively bound to Cbl.

The co-immunoprecipitation of a 170-kDa tyrosine-phosphorylated protein with Cbl in EGF-treated 293 cells but not in serum-starved cells (lanes 1, 2, 5 and 6) suggests that this protein might be the EGF receptor itself. Immunoprecipitation of 293 cell lysates with Cbl antiserum adsorbed a 170-kDa tyrosine-phosphorylated band (Fig. 3, upper panel, lane 8) that blotted faintly to EGF receptor antiserum from activated cells, but not with normal serum or from nonactivated cells (middle panel, lanes 5–8). The low signal may be due to the weaker recognition of tyrosine-phosphorylated receptors compared to nonphosphorylated receptors by the EGF receptor antibody on Western blots (compare lanes 4 and 5). EGF receptor immunoprecipitates showed a phosphotyrosine band at 120 kDa in activated (lanes 5) but not in serum-starved cells (lane 4). Blotting with Cbl antibody confirmed that the 120-kDa band in EGF receptor immunoprecipitates is Cbl (lanes 3–6, lower panel). The absence of EGF receptor protein bound to anti-Cbl antibody and of Cbl protein in anti-EGF receptor immunoprecipitates of lysates of untreated 293 cells demonstrates EGF-mediated recruitment of Cbl to tyrosine-phosphorylated EGF receptors. Comparison by densitometry of the amount of EGF receptor species in anti-Cbl immunoprecipitates versus EGF receptor immunoprecipitates indicates that approximately 30% of the total cellular EGF receptors associate with Cbl. This same value is obtained by analysis of either anti-EGF receptor or anti-tyrosine phosphorylation immunoblots (lanes 5 and 8). The large fraction of Cbl bound to the EGF receptor upon activation suggests an important functional relevance.

In EGF-activated fibroblasts, a 170-kDa protein appearing in Grb2 and Sos immunoprecipitates has been identified as the EGF receptor (5, 19, 25, 26), and it has been proposed that a complex of Sos, Grb2, and the EGF receptor is formed which leads to increased membrane localization and subsequent Ras activation. We therefore tested whether Grb2-Cbl complexes might be recruited to EGF receptors in an analogous manner. Blotting of the resolved proteins present in the anti-Cbl and anti-EGF receptor immunocomplexes shown in Fig. 3 with Grb2 mAb (bottom panel) revealed that Grb2 was bound to the EGF receptor in cell lysates from EGF-activated cells (lane 5),
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Fig. 3. Activation of 293 cells with EGF induces the association of the EGF receptor with Grb2 and Cbl. Total lysates from serum-starved (−) or activated (+) cells are shown in lanes 1 and 2. Lysates were preclarified with normal serum and immunoprecipitated with normal (lanes 3 and 6), anti-Cbl (lanes 7 and 8), or anti-EGF receptor antibody (lanes 4 and 5). Following electrophoresis and transfer to nitrocellulose, filters were probed with the antiphosphotyrosine antibody 4G10 (top), anti-EGF receptor, anti-Cbl, or anti-Grb2 (bottom), and visualized by chemiluminescence. Molecular mass markers (kDa) are shown at right.

but not from unstimulated cells (lane 4). Cbl immunoprecipitates, on the other hand, contained Grb2 in the basal state (lane 7), and EGF modestly increased the amount bound (lane 8). An increase in the Grb2/Cbl ratio was seen in several experiments (see Fig. 4). A constitutive association with Cbl via the NH₂-terminal SH3 domain of Grb2 has also been found in Jurkat cells (6, 7). Taken together, the data in Fig. 3 support the hypothesis that EGF receptor tyrosine phosphates recruit the SH2 domain of Grb2 proteins that are bound to Cbl through their NH₂-terminal SH3 domain. In addition, the activation-dependent increase of Grb2 complexed to Cbl supports our previous observation that the SH2 domain of Grb2 proteins can also bind directly to tyrosine-phosphorylated Cbl, despite the lack of a consensus YXXN motif (6, 15, 27). It is not clear from our data whether a single Grb2 protein can bind Cbl with both its SH2 and SH3 domains or whether some Grb2 proteins bind through SH2 and some through their SH3 domain.

In order to test more definitively whether the proline-rich regions of Cbl are required for binding Grb2 and the EGF receptor, peptide-mediated dissociation of Cbl-Grb2 and Cbl-EGF receptor complexes was attempted. A peptide containing the proline-rich sequence (PPVPPR) corresponding to amino acids 494–499 of Cbl was added to lysates from EGF-activated 293 cells, followed by immunoprecipitation with Cbl antiserum. Fig. 4 shows that the EGF receptor (top panel) and Grb2 (bottom panel) were both dissociated from Cbl to the same extent as the peptide concentration was increased. A Western blot (Fig. 4, middle panel) confirms that the same amount of Cbl was immunoprecipitated at all peptide concentrations. Quantitation by densitometry of the relative amount of Grb2 and EGF receptor bound to Cbl in the presence of the proline-rich peptide (Fig. 5, closed symbols) revealed that the approximate half-maximal dissociation of the EGF receptor and Grb2 from Cbl occurs at 50 μM peptide. When a nonrelevant peptide was added (open symbols), no dissociation of Grb2 or EGF receptor from Cbl was observed. These results are consistent with EGF-induced coupling of the EGF receptor to Cbl via the SH2 and SH3 domains of Grb2. However, we cannot exclude the possibility that some other SH3-containing adaptor molecule may link the EGF receptor to Cbl in this system.

A simple hypothesis that explains the data presented here is that Grb2-Cbl complexes are recruited to tyrosine phosphorylation sites on the activated EGF receptor, and that tyrosine phosphorylation of Cbl results from its juxtaposition with the receptor kinase. This hypothesis predicts that Cbl may be recruited to complexes that include other proteins that bind the EGF receptor, such as Shc proteins. Shc SH2 and phosphotyrosine-interacting domains bind EGF receptor tyrosine phosphates, and Shc itself becomes tyrosine-phosphorylated (3, 4, 20, 28). Consistent with the above prediction, we observe Shc protein in immunoprecipitates of anti-Cbl antibody when lysates from EGF-treated 293 cells are used.2 Nonetheless, further experiments will be necessary to unequivocally test whether Cbl recruitment to EGF receptors is actually necessary for its tyrosine phosphorylation. Cells expressing mutant EGF receptors missing autophosphorylation sites fail to show a pronounced block in signaling (29). Li et al. (29) have thus proposed that the EGF receptor may signal like the insulin receptor, in that SH2 domain-containing proteins are not directly bound, but rather receptor autophosphorylation causes the tyrosine phosphorylation of the docking proteins IRS-1 and Shc, which then serve as binding sites for SH2 proteins. For example, following insulin stimulation, Shc becomes rapidly tyrosine-phosphorylated, leading to Grb2-SH2 domain binding (30). It will be important in future studies to test whether tyrosine phosphorylation of Cbl is normal in response to EGF receptors with ablated tyrosine phosphorylation sites.

The multiple potential tyrosine phosphorylation sites within the protein sequence of Cbl suggest it might serve to recruit multiple signaling proteins containing SH2 or phosphotyrosine-interacting domains. In this sense, it may function similarly to IRS-1, which becomes tyrosine-phosphorylated at many sites in 2 H. Meisner and M. P. Czech, unpublished data.
response to insulin and binds SH2 domain-containing proteins (16). Upon growth factor stimulation, both proteins become transiently associated with higher concentrations of SH3 and SH2 domain proteins, including Grb2, Nck, and PI 3-kinase (6, 7, 10, 16). Interestingly, we observe several tyrosine-phosphorylated proteins that co-immunoprecipitated with Cbl in both Jurkat and 293 cells (Fig. 2, lanes 5, 9, and 10). One of these appears to be the tyrosine kinase Fyn, which binds in vitro to a 120-kDa tyrosine-phosphorylated protein through SH2 and SH3 domains (31, 32). Evidence suggests that the formation of transient complexes of Cbl with SH2 domain proteins regulates cell growth (9). This may be achieved by allowing localized increases in the activity of those proteins that bind via SH2-phosphotyrosine interaction, analogous to PI 3-kinase and Syp phosphatase interactions with phosphopeptides (14, 33). Such localized increases in specific activity may be sufficient to promote signaling pathways. In support of this, we have noted a 4-fold increase in PI 3-kinase bound to Cbl upon T-cell receptor cross-linking in Jurkat T-cells (6). It is not clear whether PI 3-kinase is recruited to Cbl in 293 cells because activity of this enzyme is very low in this model system (34). In any case, the findings presented here showing recruitment of Cbl to EGF receptor complexes and its marked tyrosine phosphorylation in response to EGF suggest that Cbl may play a key role in EGF receptor signaling.

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Note Added in Proof—After the preparation of this report, two laboratories have demonstrated that Cbl becomes tyrosine-phosphorylated by EGF in fibroblasts overexpressing the EGF receptor (35, 36). Galisteo et al. (36) conclude that Cbl is not complexed to Grb2 and binds directly to the EGF receptor in HER14 cells. The results presented here and elsewhere (6, 7) document a stable Grb2-Cbl complex in Jurkat and 293 cells, suggesting that two mechanisms may operate to couple EGF receptors to Cbl.

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