

Tyrosine Phosphorylation of Cbl upon Epidermal Growth Factor (EGF) Stimulation and Its Association with EGF Receptor and Downstream Signaling Proteins*

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We and others have shown that Cbl, the protein product of the *c-cbl* proto-oncogene, is an early target of tyrosine phosphorylation upon stimulation through the immune cell surface receptors, which signal through noncovalently associated cytoplasmic tyrosine kinases. Using human mammary epithelial cells that express a natural epidermal growth factor (EGF) receptor and require EGF as an essential growth factor, we demonstrate here that Cbl is a prominent target of tyrosine phosphorylation upon stimulation through the EGF receptor tyrosine kinase. Phosphorylation of Cbl was EGF dose-dependent, rapid (detectable as early as 5 s and maximal by 2 min), and relatively sustained (detectable even after 1 h). Co-immunoprecipitation studies demonstrated that Cbl became associated with the EGF receptor in an EGF-dependent manner. Cbl was basally associated with the adaptor protein growth factor receptor-binding protein 2 (Grb2), and this interaction was further enhanced by EGF stimulation; however, the interaction was entirely mediated via the Grb2 Src homology 3 (SH3) domains, suggesting that binding of Grb2 SH2 domain to EGF receptor provides one mechanism of Cbl's association with the EGF receptor. EGF stimulation also induced the association of Cbl with Src homology and collagen (Shc) protein, p85 subunit of the phosphatidylinositol 3-kinase and Crk proteins, in particular with the CrkL isoform. Interactions of Cbl with the EGF receptor and multiple downstream signaling proteins suggest a role for this proto-oncogene product in mitogenic signaling through growth factor receptor kinases.

Tyrosine phosphorylation provides a key switch to regulate cellular activity in response to extracellular stimuli. Many growth factor receptors, such as the epidermal growth factor receptor (EGFR)¹, possess intrinsic tyrosine kinase domains,

and their ligand-induced autophosphorylation creates docking sites for Src homology 2 (SH2) domains of cytoplasmic signaling proteins. Some signaling proteins, such as the phospholipase C γ 1, are directly phosphorylated by the receptor tyrosine kinases (RTKs) which results in the activation of their enzymatic activity. Other SH2-containing proteins serve as adaptors. For example, the growth factor receptor-binding protein 2 (Grb2) recruits guanine nucleotide exchanger son-of-sevenless protein to activated EGFR in proximity with the membrane-associated Ras. Finally, the cytoplasmic tyrosine kinases, such as the Src family members Src and Fyn, are also recruited in this manner, presumably to phosphorylate downstream effectors (1–6). Therefore, identification of additional cellular proteins that serve as substrates for RTKs is likely to enhance our understanding of how growth factors induce mitogenesis.

The protein product of the *c-cbl* proto-oncogene, p120^{cbl} (Cbl), has recently emerged as a prominent substrate of tyrosine phosphorylation downstream of the immune cell surface receptors that signal through noncovalently associated cytoplasmic tyrosine kinases (7–13). Cbl was initially identified as the cellular homolog of the *v-cbl* retroviral oncogene that induces pre-B and myeloid leukemias in mice (14). Cbl possesses a highly proline-rich region and a large number of potential tyrosine phosphorylation sites (14), which could mediate its interactions with the SH3 and SH2 domains of signaling proteins, respectively. Consistent with this possibility, Cbl forms *in vivo* complexes with Grb2 by binding to its SH3 domains, and this fraction of cellular Cbl is a target of rapid tyrosine phosphorylation in lymphocytes (9, 10, 12, 15). In addition, tyrosine-phosphorylated Cbl associated with the p85 subunit of phosphatidylinositol (PI) 3-kinase via a primarily SH2-dependent interaction (9, 12, 15, 16). Cbl was also shown to associate with the Nck adaptor protein by binding to its SH3 domains (17). Given the ubiquitous expression of signaling proteins that associate with Cbl, the well recognized roles of several of these proteins downstream of the RTKs, and the oncogenicity of altered forms of Cbl, we examined the possible involvement of this novel signal transduction protein downstream of the growth factor RTKs. Here, we used human mammary epithelial cells (MECs), which express a natural EGFR and require EGF as an essential growth factor, to demonstrate that Cbl is a prominent substrate of tyrosine phosphorylation upon EGF stimulation and that it associates with the EGFR as well as multiple signaling proteins that are recruited into EGFR signaling. These results strongly suggest a role for Cbl in signal-

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¹ The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; Grb2, growth factor receptor-binding protein 2; Shc, Src

homology and collagen protein; PI, phosphatidylinositol; RTK, receptor tyrosine kinase; SH2/3, Src homology 2/3; MEC, mammary epithelial cell; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; Tyr(P), phosphotyrosine.

ing downstream of the EGFR, perhaps by participating in a function(s) shared with other tyrosine kinase-associated cell surface receptors.

MATERIALS AND METHODS

Antibodies—The following antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): murine anti-EGFR monoclonal antibody (IgG2a, sc-120, used for immunoprecipitation), rabbit anti-EGFR (sc-003, used for immunoblotting), rabbit anti-Cbl (sc-170), rabbit anti-Grb2 (sc-255), and rabbit anti-CrkL (sc-319). The following antibodies were from Transduction Laboratories (Lexington, KY): rabbit anti-Shc (S14630); anti-Crk monoclonal antibody (S12620, reacts with Crk I and II with minimal reactivity to CrkL) (13); and rabbit anti-p85 PI 3-kinase α and β (P13030). 4G10 (anti-Tyr(P); IgG2a) (18) was kindly provided by Dr. Brian Druker (Oregon Health Science University, Portland, OR). Normal rabbit serum was used as a negative control.

Glutathione S-Transferase (GST) Fusion Proteins—Expression of human Fyn-SH3 as a COOH-terminal fusion with GST in pGEX-2T.K vector has been described (7, 19). The wild-type murine Grb2 (20) and its polymerase chain reaction-derived mutants expressed in pGEX-3X vector (Pharmacia Biotech Inc.) have been described previously (9). The Grb2 SH2 mutant (R86K) is depicted as 3-2*3, whereas Grb2 with mutations in both SH3 domains (P49L/P206L) is depicted as 3*-2-3*. Fusion proteins were affinity-purified on glutathione-Sepharose beads (Pharmacia) and quantitated, as described previously (7, 19).

Cells—EGF-dependent mammary-derived normal human mammary epithelial cell strain 76N (21) and its immortalized derivative obtained by transfection with the human papilloma virus E6 gene, and designated 16E6-P (22), were maintained in the DFCI-1 medium (21) or DFCI-1 medium containing 150 μ g/ml geneticin (G418; Life Technologies, Inc.), respectively.

Activation of Cells with EGF—Cells were plated in DFCI-1 medium in 100-mm diameter Petri dishes and grown to 50–70% confluence, followed by EGF deprivation for 3 days by culture in medium lacking EGF and insulin (23, 24). Cells were activated by the addition of EGF (100 ng/ml or as indicated; Sigma) for the indicated time points. Medium was then aspirated, and cells were quickly chilled on an ice water bath. Cells were lysed by adding 1 ml/dish of cold lysis buffer (0.5% Triton X-100 (Fluka), 50 mM Tris, pH 7.5 (at 25 °C), 150 mM sodium chloride, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml of leupeptin and pepstatin, 1 mM sodium orthovanadate, and 10 mM sodium fluoride).

Immunoprecipitations and Gel Electrophoresis of Proteins—Antibodies were added to lysates (typically from half a dish) previously pre-cleared with *Staphylococcus aureus* Cowan I strain (Pansorbin, Calbiochem). After 1–2 h of rocking at 4 °C, 20 μ l of protein A-Sepharose 4B beads (Pharmacia) were added and incubation continued for 45–60 min. Beads were washed six times in lysis buffer, and bound proteins were solubilized in Laemmli sample buffer with 2-mercaptoethanol, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblotting.

Immunoblotting—Polypeptides were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA), which were blocked at room temperature with 2% gelatin (Bio-Rad) in 10 mM Tris, pH 8, 150 mM NaCl, 0.05% Tween 20 (Bio-Rad) (TBS-T) for 1 h to overnight, incubated with optimal concentrations of primary antibodies in TBS-T for 1 h at room temperature, and washed six times in TBS-T. Filters were then incubated with horseradish peroxidase-conjugated protein A (Cappel-Organon Technika, Durham, NC), followed by visualization by enhanced chemiluminescence (ECL) according to manufacturer's recommendations (DuPont NEN). For repeated immunoblotting, membranes were stripped in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 0.1 M 2-mercaptoethanol for 30–45 min at 50 °C, rinsed in TBS-T, and blocked with gelatin/TBS-T prior to reprobing with antibodies.

Binding of Cellular Polypeptides to GST Fusion Proteins—10 μ g of purified GST fusion proteins noncovalently coupled to glutathione-Sepharose beads were rocked with Triton X-100 lysate of cells from half of a 100-mm diameter dish for 1 h at 4 °C and washed six times in lysis buffer. Bound proteins were solubilized, resolved by SDS-PAGE, and subjected to immunoblotting, as described above.

RESULTS AND DISCUSSION

We and others have recently implicated the product of *c-cbl* proto-oncogene (14, 25, 26) in signal transduction downstream of the lymphocyte antigen receptors and other immune cell surface receptors coupled to nonreceptor tyrosine kinases (7–

10, 12, 13, 15–17, 19). To fully define the potential roles of Cbl, it is important to assess whether it functions only downstream of the hematopoietic cell receptors or more generally downstream of the tyrosine kinase-coupled receptors, as suggested by its expression in nonhematopoietic tissues and cell lines (26, 27) and its association with widely expressed signaling proteins, such as Grb2, Shc, PI 3-kinase p85, Nck, and Crk proteins (7–10, 12, 13, 15–17, 19). To address the possible role of Cbl in nonhematopoietic cell signaling, we examined if it was involved downstream of the EGFR, a well characterized growth factor receptor tyrosine kinase. For this purpose, we utilized a nontumorigenic human papilloma virus E6-immortalized human mammary epithelial cell line 16E6-P and its normal parental cell strain 76N (21, 22). In both of these cells, a naturally expressed EGFR mediates an essential growth signal, as demonstrated by their inability to grow in an EGF-deficient medium, in contrast to their normal growth in EGF-supplemented medium (21, 22). To minimize basal tyrosine phosphorylation due to growth factors in their regular culture medium, cells were cultured for 3 days prior to EGF stimulation in the absence of EGF and insulin (which activates another receptor tyrosine kinase).

To assess the tyrosine phosphorylation of Cbl upon EGF stimulation of MECs, we carried out anti-Cbl immunoprecipitations from lysates of unstimulated or EGF-stimulated 16E6-P cells and analyzed these by anti-Tyr(P) immunoblotting (Fig. 1A). Relatively little basal tyrosine phosphorylation was observed in anti-Cbl immunoprecipitates from unstimulated cells (Fig. 1A, lane 1). Intense phosphorylation of a 120-kDa polypeptide, representing Cbl, was observed within 5 s of EGF stimulation, reached a maximum by 2 min, with only slightly lower signals at 60 min (last time point analyzed) (Fig. 1A, lanes 2–10). Anti-Cbl reprobing confirmed that equal amounts of Cbl protein were immunoprecipitated at each time point (Fig. 1A, lower panel). A 175–180-kDa polypeptide was observed in anti-Tyr(P) blot of anti-Cbl immunoprecipitates, and kinetics of its phosphorylation was similar to that of Cbl. As shown in Fig. 4, this polypeptide represents the EGFR.

Concurrent anti-Tyr(P) immunoblotting of the whole cell lysates revealed that the 175–180-kDa EGFR polypeptide was heavily tyrosine-phosphorylated upon EGF stimulation (Fig. 1B). Aside from the EGFR, the 120-kDa polypeptide was one of the most prominent phosphotyrosyl proteins induced by EGF. The kinetics of phosphorylation of the 120- and 175-kDa bands in whole cell lysates was similar to that noted in anti-Cbl immunoprecipitates (compare Fig. 1, A with B). Thus, EGF stimulation of 16E6-P cells induces a prominent, rapid, and sustained tyrosine phosphorylation of Cbl.

To further characterize the EGF-induced Cbl tyrosine phosphorylation, we carried out anti-Cbl and anti-EGFR immunoprecipitations from 16E6-P cells stimulated with different concentrations of EGF. Anti-Tyr(P) immunoblotting revealed a dose-related increase in Cbl tyrosine phosphorylation with 1–100 ng/ml EGF (Fig. 2); a small increase was detected with 0.1 ng/ml EGF upon longer exposure of the blot (not shown). For comparison, a dose-related increase in EGFR phosphorylation was also noted with 0.1–100 ng/ml EGF, with associated proteins being detectable primarily at higher (1–100 ng/ml) EGF concentrations. Relatively equal loading of immunoprecipitates in various lanes was revealed by anti-Cbl or anti-EGFR immunoblotting.

To rule out the possibility that Cbl phosphorylation in 16E6-P cells was related to their papilloma virus oncogene-induced immortalization, we also examined the 76N normal MECs from which the 16E6-P cell line was derived. A clear EGF-dependent tyrosine phosphorylation of Cbl and its asso-

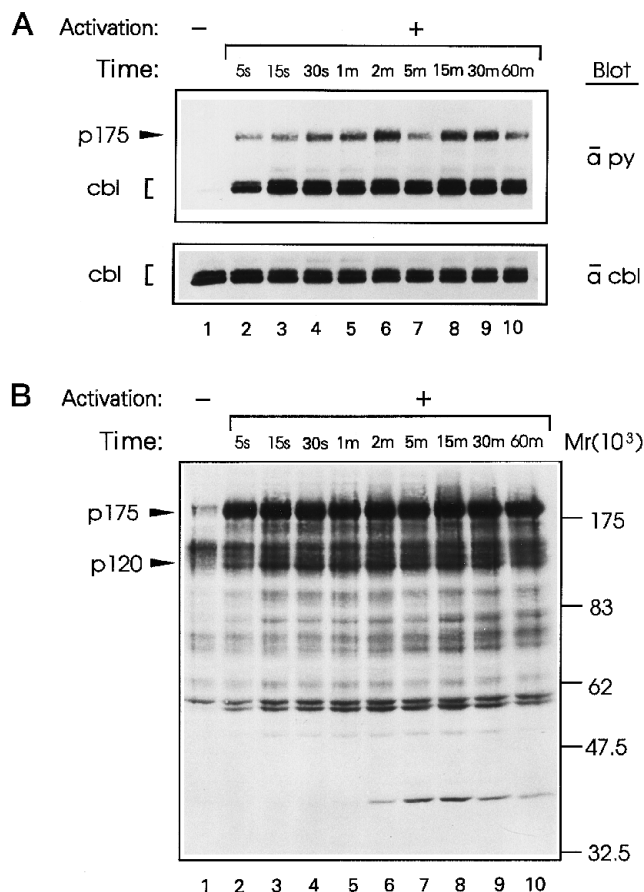


FIG. 1. EGF induces rapid and sustained tyrosine phosphorylation of Cbl in EGF-dependent human mammary epithelial cell line 16E6-P. A, cells were EGF-deprived for 3 days by growth in EGF-free medium. Cells were then left unstimulated (–) or were stimulated with 100 ng/ml EGF for the indicated time points (s, seconds; m, minutes) followed by lysis in Triton X-100 lysis buffer. Anti-Cbl immunoprecipitations, each from lysate of one 100-mm diameter plate, were resolved by SDS-9%PAGE, transferred to PVDF membrane, and immunoblotted with anti-Tyr(P) antibody (upper panel). Membrane was then reacted with protein A-horseradish peroxidase conjugate, and ECL detection was used to visualize the immunoprecipitated species. The filter was stripped and re-blotted with anti-Cbl antibody to show equal Cbl immunoprecipitation (lower panel). Cbl and associated Tyr(P) protein, p175 (EGFR; see Fig. 4), are indicated on left. B, 1/20th of the cell lysates used in Fig. 1A were directly analyzed by anti-Tyr(P) immunoblotting. p120 (Cbl) and p175 (EGFR) are indicated on the left. Reprobing of this filter with anti-Cbl (not shown) demonstrated equal loading.

ciation with EGFR was observed in 76N cells similar to that seen with 16E6-P cells (Fig. 3). Cbl phosphorylation was also observed using an EGF-dependent primary mammary tumor cell line 21PT (28) (data not shown).

Coimmunoprecipitation of a 175-kDa phosphotyrosyl protein, comigrating with the EGFR, in anti-Cbl immunoprecipitates (Figs. 1A, 2 and 3) suggested that these proteins associate upon EGF stimulation. To characterize this association further, we carried out reciprocal immunoblotting of their immunoprecipitates. Anti-Tyr(P) immunoblotting of the anti-EGFR immunoprecipitates revealed a prominent EGF-dependent tyrosine phosphorylation of the major 175–180-kDa EGFR polypeptide as well as several associated polypeptides migrating at 120, 75, 66, 52, and 48 kDa (Fig. 4, lanes 3 and 4). Based on comigration and immunoblotting, the 66-, 52-, and 48-kDa polypeptides correspond to Shc proteins (Fig. 4, compare lanes 7–10 with lanes 3 and 4). The EGFR-associated 120-kDa phosphotyrosyl polypeptide comigrated with directly immunoprecipitated Cbl (Fig. 4, compare lanes 4 and 6) and was immunoblotted by anti-Cbl antibody (see anti-Cbl blot). The major

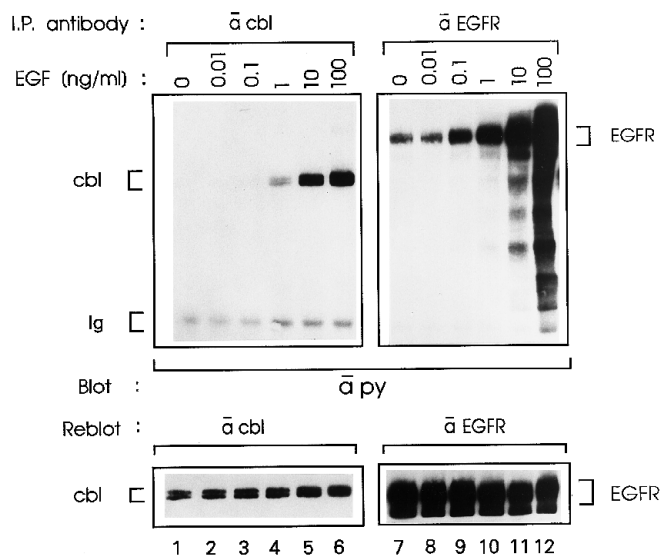


FIG. 2. EGF dose response of Cbl and EGFR tyrosine phosphorylation in 16E6-P cells. EGF-deprived 16E6-P cells were stimulated with indicated concentrations of EGF for 10 min, and lysates (from half of a 100-mm diameter dish) were immunoprecipitated (I.P.) with anti-Cbl (left panels) or anti-EGFR (right panels) antibody followed by anti-Tyr(P) immunoblotting using protein A-horseradish peroxidase conjugate and ECL detection. Filters shown on top were stripped and re-blotted with anti-Cbl (left) or anti-EGFR antibody (right), respectively, and show equal loading of immunoprecipitated material in all lanes. Cbl, Ig heavy chain (Ig), and the EGFR are indicated.

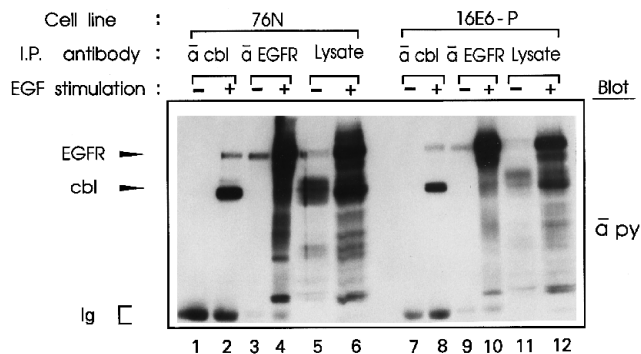


FIG. 3. EGF-dependent tyrosine phosphorylation of Cbl in untransformed primary mammary epithelial cell strain 76N, the parental line for immortal 16E6-P cells. EGF-deprived cells were either left unstimulated (–) or were stimulated with 100 ng/ml EGF for 10 min. Lysates (from half a 100-mm plate) were immunoprecipitated with the indicated antibodies (I.P.) and resolved next to 1/20th of the whole cell lysates, followed by anti-Tyr(P) immunoblotting using ECL detection. Locations of EGFR, Cbl, and Ig heavy chain (Ig) are indicated. The identity of a polypeptide that migrates just above Cbl and is seen in unstimulated and stimulated lanes is unknown.

Cbl-associated phosphotyrosyl polypeptide at 175–180 kDa precisely comigrated with directly immunoprecipitated EGFR. Longer exposures of anti-EGFR immunoblot (not shown) revealed that the Cbl-associated 175-kDa polypeptide was reactive with anti-EGFR antibody. Cbl-EGFR association was also observed by performing anti-tag immunoprecipitations from a MEC transfectant expressing an influenza hemagglutinin-tagged Cbl (data not shown). Altogether, the analyses presented above clearly demonstrate that Cbl is a substrate of tyrosine phosphorylation upon EGF stimulation of human mammary epithelial cells and forms a protein complex with the EGFR in an EGF-dependent manner. These results confirm and extend similar results in other cell types that were published while our manuscript was in preparation and review (29–33).

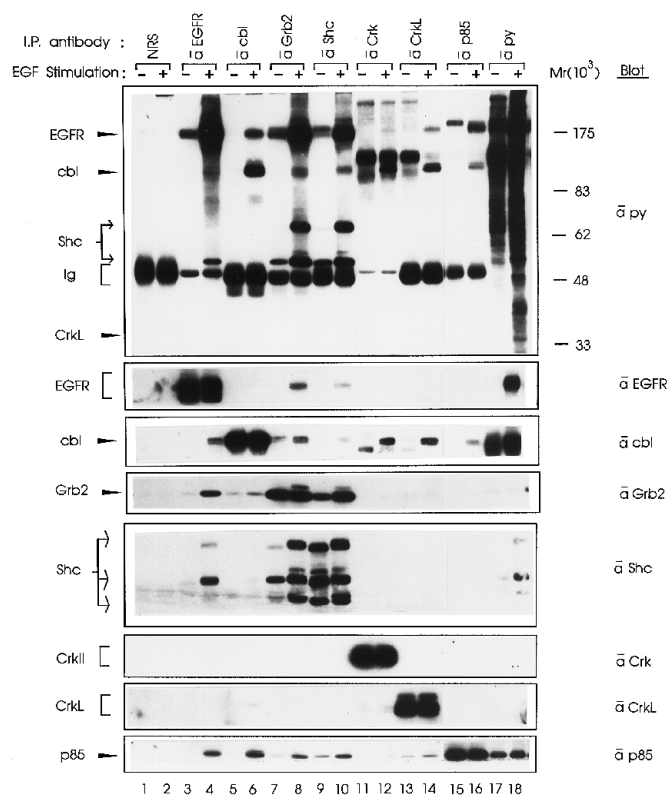


FIG. 4. *In vivo* association of Cbl with the EGFR, Grb2, Shc, and Crk proteins and the p85 subunit of PI 3-kinase in 16E6-P mammary epithelial cells. Immunoprecipitations were carried out with indicated antibodies (I.P. antibody) from lysates of unstimulated cells (–) or cells stimulated for 10 min with 100 ng/ml EGF (+). Proteins were resolved by SDS-PAGE, transferred to PVDF membrane, immunoblotted with anti-Tyr(P) antibody followed by protein A-horseradish peroxidase conjugate, and detected by ECL (upper panel). The membrane was then cut into different size regions, stripped, and serially probed as appropriate with antibodies shown on right. Locations of identified polypeptides are shown on left. Anti-Crk blotting showed equal amounts of Crk II (shown) as well as Crk I (not shown) in lanes 11 and 12. Ig, Ig heavy chain.

Previous studies have demonstrated that EGF-dependent association of cytoplasmic signaling proteins with the EGFR is mediated by binding of their SH2 domains to phosphotyrosyl peptide motifs on the autophosphorylated receptor (1–6). Cbl lacks an SH2 domain, suggesting that its interaction with the EGFR may be mediated either through SH2 domain-containing adaptor proteins or a novel SH2-independent mechanism. Grb2 and Nck represented two likely candidates to serve an adaptor role, since they are known to bind to proline-rich sequences within Cbl through their SH3 domains (7–10, 12, 15–17, 19) and to tyrosine-phosphorylated EGFR via their SH2 domains (1–6, 34). To assess this possibility, an immunoprecipitation-immunoblotting strategy was employed.

As expected, Grb2 showed a prominent EGF-dependent association with tyrosine-phosphorylated EGFR and Shc proteins (Fig. 4, lanes 7 and 8 versus lanes 3 and 4 or 9 and 10). In addition, a prominent 120-kDa phosphotyrosyl polypeptide which comigrated with Cbl (Fig. 4, compare lanes 6 and 8) was coimmunoprecipitated with Grb2. Direct anti-Cbl immunoblotting revealed this polypeptide to be Cbl (Fig. 4, anti-Cbl blot, lane 8). Reciprocal coimmunoprecipitation of Grb2 with Cbl was also observed (Fig. 4, anti-Grb2 blot, lane 6). Grb2-Cbl association was observed both in unstimulated and EGF-stimulated cells. However, in contrast to T cells, where similar levels of Grb2 associated with Cbl before and after activation (9), Cbl-Grb2 interaction in 16E6-P cells was modestly in-

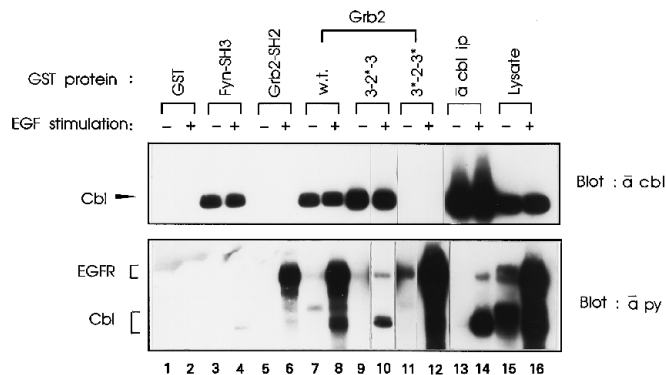


FIG. 5. Binding of Cbl to GST fusion proteins of Grb2 is exclusively mediated through Grb2 SH3 domains. Cell lysates from one-half of a 100-mm diameter plate of unstimulated cells (–) or cells stimulated with 100 ng/ml EGF for 10 min (+) were incubated for 1 h with GST fusion proteins noncovalently immobilized on glutathione-Sepharose beads (5 μ l of packed beads; 10 μ g of fusion protein; total volume, 1 ml), and bound proteins were solubilized in sample buffer. Binding reactions or whole cell lysate (from 1/20th of a plate) were resolved by SDS-PAGE and subjected to anti-Cbl immunoblotting (upper panel) using protein A-horseradish peroxidase conjugate and ECL detection. w.t., wild-type Grb2. 3-2-3 refers to NH₂-terminal SH3 and SH2 and COOH-terminal SH3 domains of Grb2. Asterisks denote mutated domains. Mutated residues were: NH₂-terminal SH3, P49L; SH2, R86K; COOH-terminal SH3, P206L. The filter was stripped and immunoblotted with anti-Tyr(P) antibody (lower panel). Cbl and EGFR are indicated on left. α cbl i.p. (lanes 13 and 14), immunoprecipitation from the same amount of cell lysate as used for binding reactions.

creased by EGF treatment (Fig. 4, anti-Cbl blot, lanes 7 and 8; anti-Grb2 blot, lanes 5 and 6).

To elucidate the nature of Grb2-Cbl interaction in EGF-stimulated MECs, *in vitro* binding analyses were performed using GST fusion proteins of Grb2 carrying mutations in SH2 domain or in both SH3 domains. Lysates of unstimulated or EGF-stimulated 16E6-P cells were incubated with bead-immobilized GST fusion proteins, and bound polypeptides were detected by anti-Cbl and anti-Tyr(P) immunoblotting (Fig. 5). Similar amounts of Cbl associated with the wild-type GST Grb2 fusion protein regardless of whether the lysates were derived from unstimulated or EGF-stimulated cells (Fig. 5, lanes 7 and 8), as was the case using GST Fyn-SH3 (lanes 3 and 4). Importantly, Cbl binding capacity was fully retained in GST-Grb2 fusion protein with a mutation in the SH2 domain (Fig. 5, lanes 9 and 10). Conversely, GST-Grb2 with mutations in both SH3 domains failed to bind to Cbl (Fig. 5, lanes 11 and 12); GST fusion protein of the isolated Grb2 SH2 domain also failed to bind to Cbl (lanes 5 and 6). Anti-Tyr(P) reprobing of the blot demonstrated that Cbl was tyrosine-phosphorylated upon EGF stimulation and associated with the EGFR (Fig. 5, lower panel, lanes 13 and 14). Furthermore, GST-Grb2 with a mutated SH2 (lanes 9 and 10) showed a dramatically reduced binding to the EGFR, whereas Grb2-SH2 (lanes 5 and 6) and Grb2 fusion protein with SH3 domain mutations (lanes 11 and 12) were fully active. These data demonstrate that EGF stimulation does not induce a Grb2 SH2 domain-binding site on Cbl, indicating that the Cbl-Grb2 interaction in MECs is exclusively Grb2 SH3 domain-mediated, similar to our findings in T and B cells (9, 12).

Grb2 can also associate with the EGFR indirectly, via Grb2 SH2 binding to tyrosine-phosphorylated Shc; Shc binds to the EGFR via its SH2 domain (1–6). Consistent with this scheme, EGF-dependent Grb2-Shc and EGFR-Shc associations were prominent in 16E6-P cells (Fig. 4, lanes 3 and 4 and 7 and 10). Importantly, a small amount of Cbl coimmunoprecipitated with Shc (lane 10, anti-Tyr(P) and anti-Cbl blots); a small amount of Shc was also observed in anti-Cbl immunoprecipitates upon

longer exposure of anti-Shc immunoblot (not shown). Consistent with Grb2-mediated Shc-Cbl interaction, Shc-Cbl complex was much less abundant compared with either the Shc-Grb2 or Grb2-Cbl complexes (Fig. 4), and a GST-Shc SH2 fusion protein did not bind to Cbl in either unstimulated or the EGF-stimulated cells (data not shown).

Since Nck is known to bind to activated EGFR through its SH2 domain (34) and to Cbl via its SH3 domains (17), Nck represented another potential adaptor that could mediate Cbl-EGFR association. However, anti-Nck immunoprecipitates did not reveal a detectable association with Cbl although Nck associated with the EGFR in activated 16E6-P cells (data not shown). In addition, we did not observe a complex between Cbl and Fyn, another protein that associates with Cbl via its SH3 domain in T and B cells (7, 9, 12), although a small fraction of the EGFR co-immunoprecipitated with anti-Fyn antibodies (data not shown). Altogether, the above results are consistent with a major role for Grb2 in mediating Cbl-EGFR association.

While this paper was under review, other reports on the potential mechanisms of Cbl-EGFR association have appeared. Meisner and Czech (31) showed that in human embryonic cell line 293 Cbl is constitutively associated with Grb2, and EGF stimulation induces a strong association between Cbl and the EGFR. In addition, these workers showed that both the Grb2 and Cbl were released from the EGFR by a proline-rich peptide specific for Grb2 SH3 domains. Independently, Galisteo *et al.* (30) also observed Grb2 and Shc in anti-Cbl immunoprecipitates of EGF-stimulated murine fibroblasts overexpressing the human EGFR. Thus, these studies, like ours, support the role of Grb2 in mediating Cbl-EGFR association. However, Galisteo *et al.* (30) also demonstrated a direct *in vitro* binding between the NH₂-terminal half of Cbl expressed as a GST fusion protein and the tyrosine-phosphorylated EGFR cytoplasmic tail. Notably, Bowtell and Langdon (32) expressed the NH₂-terminal portion of Cbl, devoid of its proline-rich regions, in murine fibroblasts and showed that it co-immunoprecipitated with the EGFR in unstimulated cells with a modest increase of this association upon EGF stimulation. Interestingly, this association was considerably lower compared with that observed with either the wild-type Cbl or mutants that retained the proline-rich region. Altogether, it appears likely that both the direct and adaptor-dependent interactions contribute to Cbl-EGFR association, although additional studies will be needed to clarify their relative importance particularly in the context of different cell types where the relative levels of Cbl, EGFR, and Grb2 proteins may be quite different. Independently, genetic studies in *Caenorhabditis elegans* have identified a structurally conserved Cbl homolog, sli-1, as a negative regulator of the EGFR, *let-23*, which controls vulval development through a signaling pathway involving Grb2 and Ras homologs (35).

Recent analyses in T and B cells have demonstrated an activation-dependent, primarily SH2-mediated, complex of the p85 subunit of PI 3-kinase with Cbl (9, 12, 15). Furthermore, a substantial fraction of the PI 3-kinase activity became associated with Cbl upon antigen receptor stimulation (9, 12, 15). Since PI 3-kinase is known to be recruited into EGF signaling (36), we examined if Cbl-PI 3-kinase p85 complexes are induced by EGF stimulation of 16E6-P cells. As seen in Fig. 4 (anti-p85 blot, lanes 5 and 6; anti-Tyr(P) and anti-Cbl blots, lanes 15 and 16), a prominent EGF-dependent association of Cbl and PI 3-kinase p85 was observed in 16E6-P cells. Notably, a smaller but significant EGF-dependent coimmunoprecipitation of PI 3-kinase p85 with the EGFR (lanes 3 and 4), Grb2 (lanes 7 and 8), and Shc (lanes 9 and 10) was also observed. In view of the lack of a consensus p85 SH2 domain binding motif on the EGFR (36), association of the PI 3-kinase p85 subunit with

tyrosine-phosphorylated Cbl may provide one mechanism to recruit this enzyme to the EGFR. A similar role for Cbl has been suggested by Soltoff and Cantley (33), based on their studies on PC12 and other cells.

Our recent analyses have revealed that Cbl is a major Crk- and CrkL-associated tyrosine-phosphorylated polypeptide in activated T cells (13). Since Crk proteins are widely expressed, and Crk SH2 domain can interact with the EGFR (37, 38), we examined the possible association of Cbl with Crk proteins. Anti-CrkL, and to lesser extent anti-Crk antibody (which recognizes CrkI and II) (13), co-immunoprecipitated a small amount of EGFR in EGF-stimulated 16E6-P cells (Fig. 4, lanes 11–14). In addition, a small but reproducible tyrosine phosphorylation of CrkL (Fig. 4, lane 14) and CrkII (seen upon longer exposure, not shown) was observed upon EGF stimulation. Notably, these analyses showed that Cbl was the major co-immunoprecipitating tyrosyl phosphoprotein in anti-Crk (Fig. 4, lanes 11 and 12) and in particular in anti-CrkL (lanes 13 and 14) immunoprecipitates. Anti-CrkL immunoblotting revealed that a small amount of CrkL was co-immunoprecipitated by anti-Cbl (Fig. 4, lane 6). Thus, in addition to their direct association, Crk proteins may also be recruited into EGFR signaling indirectly through their association with tyrosine-phosphorylated Cbl. We have shown previously that Cbl association with the Crk proteins is SH2 domain-mediated, which allows a ternary complex to be formed between tyrosine-phosphorylated Cbl and C3G, a guanine nucleotide exchange factor for the small GTP-binding protein Rap 1A that is constitutively associated with the SH3 domains of Crk proteins (13, 39). Thus, Cbl-Crk interaction could provide an alternate mechanism to recruit this novel signaling pathway to the EGFR.

In conclusion, we show that Cbl is a major substrate for the EGFR, associates with this receptor upon EGF stimulation, and forms complexes with several signaling proteins that play key roles in EGF-mediated cell growth. Identification of Cbl proto-oncoprotein as a downstream element in signaling through both the hematopoietic and nonhematopoietic cell receptors suggests an important role for this protein. The present system, in which EGF mediates an essential growth signal, should complement other cell systems to more precisely define the biological function(s) of Cbl.

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