p120<sup>cbl</sup> Is a Cytosolic Adapter Protein That Associates with Phosphoinositide 3-Kinase in Response to Epidermal Growth Factor in PC12 and Other Cells*

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Although epidermal growth factor (EGF) activates phosphoinositide (PI) 3-kinase activity in a number of types of cells or cell lines, in most cases that we have investigated the p85 regulatory subunit of PI 3-kinase does not appear to bind directly to the EGF receptor. Previously we demonstrated that EGF-dependent activation of PI 3-kinase activity in A431 cells is accompanied by the binding of p85 to ErbB3, an EGF receptor homologue. However, this mechanism did not explain the large activation of PI 3-kinase activity that was found in PC12 and A549 cells, which possess little or no ErbB3. Here we provide evidence that the p120<sup>cbl</sup> proto-oncoprotein is an intracellular adapter protein that associates with PI 3-kinase and thus is involved in the EGF-dependent activation of this enzyme in these two cell lines. Using an anti-p120<sup>cbl</sup> antibody, we immunoprecipitated the EGF receptor from PC12 cells and PI 3-kinase activity from PC12 and A549 cells in an EGF-dependent fashion. Treatment of PC12 cells with nerve growth factor or insulin stimulated large increases in PI 3-kinase activity that was immunoprecipitated using anti-Tyr(P) antibody but not using anti-p120<sup>cbl</sup> antibody. In EGF-treated PC12 cells, the tyrosine phosphorylation of p120<sup>cbl</sup> displayed similar kinetics to the activation of PI 3-kinase as measured by both in vivo lipid production and lipid kinase assays conducted using anti-p120<sup>cbl</sup> and anti-Tyr(P) immunoprecipitates. The use of glutathione S-transferase fusion proteins of various domains of p85 demonstrated that p120<sup>cbl</sup> associated with both the SH2 and SH3 domains of p85. p120<sup>cbl</sup> was also present in A431 cells and offers an additional pathway by which EGF can activate PI 3-kinase in these cells.

In a previous study we demonstrated that EGF<sup>1</sup>-stimulated PI 3-kinase activity in A431, PC12, and A549 cells was immunoprecipitated using an anti-Tyr(P) antibody (Soltoff et al., 1994). In addition to the EGFR, A431 cells express ErbB3, a ~180-kDa protein that heterodimerizes with the EGFR in response to EGF. We demonstrated that ErbB3 serves as an adapter protein between the EGFR and PI 3-kinase in A431 cells, and EGF-dependent PI 3-kinase activity was immunoprecipitated from these cells using anti-ErbB3 antibodies. ErbB3 has multiple copies of the consensus sequence for binding the SH2 domain of the p85 subunit of PI 3-kinase (Tyr-X-X-Met), and phosphorylation at these sites presumably mediates the association. However, since PC12 and A549 cells have little or low levels of ErbB3, we hypothesized that another protein must serve as an adapter between the EGFR and PI 3-kinase in these cells.

We previously observed the EGF-dependent tyrosine phosphorylation of a ~120–130-kDa protein in PC12 and A549 cells (Soltoff et al., 1994). The cbl (c-cbl) proto-oncogene encodes a 120-kDa protein (p120<sup>cbl</sup>) that is localized exclusively in the cytoplasm. This protein was recently shown to be phosphorylated on tyrosine in response to activation of the T cell antigen receptor (Donovan et al., 1994). The overexpression of p120<sup>cbl</sup> does not lead to transformation. The protein encoded by a retrovirus version of c-cbl (v-cbl) has 60% of the C terminus deleted and is located both in the cytoplasm and in the nucleus (Blake et al., 1991, 1993). The latter localization was correlated with the transforming ability of cbl.

The p120<sup>cbl</sup> protein has a Tyr-X-X-Met site that could interact with the SH2 domains of p85 if phosphorylated and a proline-rich sequence that could bind to the SH3 domain of p85. Thus, we investigated the possibility that p120<sup>cbl</sup> binds PI 3-kinase in response to stimulation of PC12 or A549 cells with EGF.

**MATERIALS AND METHODS**

**Chemicals**—All chemicals were reagent grade or better. Dulbecco’s modified Eagle’s medium was obtained from Life Technologies, Inc. ([<sup>32</sup>PI]ATP (specific activity, 3,000 Ci/mmol) was purchased from DuPont NEN. EGF (catalog no. 01-107) was purchased from Upstate Biochemicals, Inc. (Lake Placid, NY).

**Antibodies**—Anti-p120<sup>cbl</sup> antibody was purchased from Santa Cruz (catalog no. SC-170). Anti-phosphotyrosine antibody was a generous gift from Dr. Thomas Roberts (Dana Farber Hospital, Boston, MA). Antibody to the p85 subunit of PI 3-kinase was raised in rabbits by Dr. Brian Schaufhausen (Tufts University, Boston, MA) and is commercially available from Upstate Biochemicals (catalog no. 06-195).

**Cell Culture**—A431, A549, and PC12 cells were grown in 100-mm diameter dishes as described previously (Soltoff et al., 1994). All cells were switched to a low serum (0.1% serum) overnight prior to exposure to growth factors. Cells were used when confluent or nearly confluent.

**Assay of PI 3-Kinase Activity**—Growth factors were added to cells for the indicated times at 37 °C. Lysis of cells, immunoprecipitations, and lipid kinase assays were performed as described previously (Soltoff et al., 1994). Exogenous phosphatidylinositol was used as a substrate in the lipid kinase assays. After extracting the lipids and separating the products using thin layer chromatography, the radiolabeled spots that co-chromatographed with PtdIns-P<sub>F</sub> were excised and quantified by scintillation counting.
**RESULTS**

**Immunoprecipitation of PI 3-Kinase Activity Using an Anti-p120<sup>cbl</sup> Antibody**—EGF-dependent PI 3-kinase activity was immunoprecipitated from PC12 cells using anti-p120<sup>cbl</sup> antibody. The time course of EGF-dependent PI 3-kinase activity in immunoprecipitates from PC12 cells was compared using anti-p120<sup>cbl</sup> and anti-Tyr(P) antibodies (Fig. 1A). In both immunoprecipitates, the activity was near maximal at ~15 s (the earliest time point), it reached a peak at 1 min, and it was sustained at a lower elevated level after 5–15 min. In agreement with our previous observations (Soltoff et al., 1994), immunoprecipitations of the EGF receptor did not have significant EGF-dependent PI 3-kinase activity. The results of these studies indicate that PI 3-kinase binds directly or indirectly to p120<sup>cbl</sup> in these cells.

A more complete time course for PI 3-kinase activity in anti-Tyr(P) immunoprecipitates from EGF-treated PC12 cells demonstrates that elevated PI 3-kinase activity was maintained for at least 60 min after initial exposure to EGF (Fig. 1B).

To investigate the alterations in the various phosphoinositide products in vivo, we measured the levels of the phosphorylated polyphosphoinositides in [<sup>32</sup>P]orthophosphate-labeled cells. The lipids were extracted and deacylated, and the glycerophospholipid polyphosphates were identified and quantified by HPLC (Fig. 1C). Within the first minute of exposure to EGF, [<sup>32</sup>P]PtdIns-3,4-P<sub>2</sub> and [<sup>32</sup>P]PtdIns-3,4,5-P<sub>3</sub> increased to 4–10 times the basal levels, and elevated levels of these lipids were maintained for at least 30 min. After the first minute of EGF exposure, the decrease in PI-3,4,5-P<sub>3</sub> levels and continued increase in PI-3,4-P<sub>2</sub> levels are probably due to the activity of a 5'-phosphatase that dephosphorylates PI-3,4,5-P<sub>3</sub> (Stephens et al., 1991; Carter et al., 1994; Jackson et al., 1995). These results indicate that the time course of the activation of PI 3-kinase in vivo is similar to that observed in the in vitro kinase assay using the anti-Tyr(P) and anti-p120<sup>cbl</sup> immunoprecipitates (Fig. 1, A and B).

A comparison of EGF-, NGF-, and insulin-stimulated PI 3-kinase activities from PC12 cells immunoprecipitated using anti-p120<sup>cbl</sup> and anti-Tyr(P) antibodies is shown in Fig. 2. All three growth factors promoted large increases in PI 3-kinase activi-
ties that were immunoprecipitable using anti-Tyr(P) antibody. However, the relative increase in PI 3-kinase activity in the anti-p120\(^{cbl}\) immunoprecipitates of EGF-treated cells, unlike those of insulin- or NGF-treated cells, was similar to the relative increase observed in anti-Tyr(P) immunoprecipitates. In addition, only in EGF-stimulated cells was the absolute amount of growth factor-stimulated PI 3-kinase activity in anti-p120\(^{cbl}\) immunoprecipitates similar to that which was measured in anti-Tyr(P) immunoprecipitates. Studies by various investigators have demonstrated that p85 associates with gp140\(^{cbl}\) and insulin receptor substrate-1 (IRS-1) in response to NGF and insulin, respectively (Soltoff et al., 1992; Sun et al., 1993). In combination with previous studies, the results in Fig. 2 indicate that each of the receptors for EGF, NGF, and insulin stimulates PI 3-kinase by phosphorylating a different adapter protein.

Since we had hypothesized that an adapter protein was also responsible for the EGF-dependent PI 3-kinase activity found in A549 cells (Soltoff et al., 1994), we investigated the possibility that PI 3-kinase associates with p120\(^{cbl}\) in this cell line. Similar to the results reported above with PC12 cells, treatment of A549 cells with EGF (100 ng/ml for 1 min) resulted in a similar increase of PI 3-kinase activity in anti-p120\(^{cbl}\) and anti-Tyr(P) immunoprecipitates (10.9 ± 4.3 (n = 3) and 7.5 ± 0.2 (n = 3) times basal activity in anti-p120\(^{cbl}\) and anti-Tyr(P) immunoprecipitates, respectively). In these experiments the EGF-stimulated PI 3-kinase activity in anti-p120\(^{cbl}\) immunoprecipitates was 62.6 ± 21.2% (n = 3) as large as that found in anti-Tyr(P) immunoprecipitates, similar to that found in PC12 cells (Fig. 2, legend). These results are consistent with p120\(^{cbl}\) serving as an adapter protein between the EGF receptor and PI 3-kinase in both PC12 and A549 cells.

EGF-dependent increases in PI 3-kinase activity were also found in anti-p120\(^{cbl}\) immunoprecipitates from A431 cells. For cells treated with EGF (100 ng/ml, 5 min), the activity in anti-p120\(^{cbl}\) immunoprecipitates was 8.3 ± 3.2 (n = 5) times that in unstimulated cells, and this value was 5.0 ± 1.8 (n = 5) in anti-Tyr(P) immunoprecipitates. These results suggest that in addition to ErbB3, which is a transmembrane receptor protein, the cytosolic protein p120\(^{cbl}\) provides another site for the recruitment of p85 in A431 cells.

EGF Promotes the Tyrosine Phosphorylation of p120\(^{cbl}\) and Its Association with p85—Anti-p120\(^{cbl}\) and anti-Tyr(P) immunoprecipitates from cells exposed to EGF were immunoblotted with various antibodies. p85, the regulatory subunit of PI 3-kinase, was identified in immunoprecipitates using either antibody (Fig. 3A). For each antibody, the peak in the immunoprecipitation of p85 was at ~1 min, consistent with the peak in PI 3-kinase activity observed in Fig. 1.

When these immunoprecipitates and accompanying lysates were reprobed for p120\(^{cbl}\), a ~12-kDa protein was identified (Fig. 3B). In the anti-Tyr(P) immunoprecipitates, the amount of p120\(^{cbl}\) present peaked at 1 min after EGF addition, in agreement with the peak in PI 3-kinase activity and p85 in anti-Tyr(P) immunoprecipitates. When these blots were reprobed for tyrosine-phosphorylated proteins, the time-dependent increases in tyrosine phosphorylation of p120\(^{cbl}\) in anti-p120\(^{cbl}\) immunoprecipitates (Fig. 3C) showed similar kinetics to increases in the amount of p120\(^{cbl}\) protein in anti-Tyr(P) immunoprecipitates (Fig. 3A), with the peak amount of protein appearing after a 1-min exposure to EGF. In cell lysates, proteins that co-localize with the EGF receptor and p120\(^{cbl}\) constitute the major proteins that are tyrosine-phosphorylated in EGF-treated PC12 cells (Fig. 3C). The EGF receptor also was immunoprecipitated from lysates of EGF-treated PC12 cells using anti-p120\(^{cbl}\) antibody (Fig. 3D).

The concentration dependence of EGF on PI 3-kinase activity immunoprecipitated using anti-p120\(^{cbl}\) antibody is shown in Fig. 4A. The PI 3-kinase activity was increased in a concentration-dependent manner between 1 and 100 ng/ml EGF (EC\(_{50}\) ~10 nM). Consistent with these results, the association of PI 3-kinase (p85) with p120\(^{cbl}\) displayed a similar concentration dependence (Fig. 4B), suggesting that PI 3-kinase binds directly to p120\(^{cbl}\) in response to EGF.

p120\(^{cbl}\) Associates with GST Fusion Proteins of p85—Lysates from PC12 cells were probed with different GST fusion proteins of p85, including the C-terminal SH2, N-terminal SH2, and SH3 domains and the full-length p85. In cells treated with EGF, p120\(^{cbl}\) associated with full-length p85 and was preferentially bound to the C-terminal rather than to the N-terminal SH2 domain. The association of p120\(^{cbl}\) with the C-terminal domain was much less than that with full-length p85 (Fig. 5). Consistent with p85 binding to SH2 domains of p120\(^{cbl}\), these interactions were dependent on EGF and occurred in parallel with the tyrosine phosphorylation of p120\(^{cbl}\). In contrast, p120\(^{cbl}\) was constitutively associated with the p85 SH3 domain, and the majority of p120\(^{cbl}\) that bound in an EGF-independent manner was not phosphorylated on tyrosine residues.

**DISCUSSION**

The results presented here demonstrate that p120\(^{cbl}\) becomes phosphorylated on tyrosine in response to EGF stimulation of PC12 cells. In addition, p120\(^{cbl}\) associates with PI 3-kinase in response to EGF stimulation. The tyrosine phosphorylation of p120\(^{cbl}\) (Fig. 3, B and C) and the association of p85 with the p120\(^{cbl}\) protein (Figs. 3A and 4B) are consistent with the SH2 domain of p85 binding to tyrosine-phosphorylated p120\(^{cbl}\) (Fig. 5). The p120\(^{cbl}\) protein has two sites (Tyr\(^{371}\) and Tyr\(^{611}\)) with the appropriate Tyr-X-X-Met motif that forms the consensus binding site for p85 (Cantley et al., 1991); it was identified as a likely binding protein for PI 3-kinase from a search of the GenBank data base for proteins containing the PI 3-kinase SH2 binding motif (Songyang et al., 1993). p120\(^{cbl}\) also has proline-rich regions that could interact with the SH3 domain of p85. The interaction of p85 with other proteins, including pp60\(^{src}\), p56\(^{lck}\), and p59\(^{fyn}\) (Liu et al., 1993; Prasad et al., 1993; Kapeller et al., 1994), through SH3-binding motifs has...
been observed, and SH3 domains of p120<sup>bl</sup> were reported to bind to p47<sup>nck</sup> (Rivero-Lezcano et al., 1994). When PC12 lysates were probed with GST fusion proteins of p85, p120<sup>bl</sup> bound to the C-terminal SH2 domain in an EGF-dependent manner and constitutively bound to the SH3 domain, suggesting that both domains contribute to the association of p85 with p120<sup>bl</sup>. The SH2 and SH3 domains of p85 also bound to p120<sup>bl</sup> in activated T cells (Fukazawa et al., 1995). The contrast between the ability of the SH3 domain of p85 to bind p120<sup>bl</sup> from unstimulated cells and the inability of full-length p85 to do this suggests that the SH3 domain may be sequestered in full-length p85. We
previously suggested that this occurs via binding to proline-rich sequences endogenous to p85 (Kapeller et al., 1994). Thus, a model consistent with the data is that engagement of the SH2 domains of p85 exposes the SH3 domain for further interaction with p120\(^{cd}\) to increase the affinity. The specific mapping of the p85 binding site on p120\(^{cd}\) will require expression of cbl mutants lacking the motifs predicted to bind these domains.

The interaction between p120\(^{cd}\) and the EGF receptor appears similar to the insulin receptor/IRS-1 interaction. IRS-1 is a cytoplasmic protein that has multiple tyrosine residues that are phosphorylated by the insulin receptor, and PI 3-kinase binds mainly to IRS-1 and not the insulin receptor upon the addition of insulin to cells (reviewed in White and Kahn (1994)). Our results suggest that p120\(^{cd}\), a cytosolic protein, binds PI 3-kinase but may not bind tightly to the EGFR. p120\(^{cd}\) may be a substrate of the EGFR, since the sequence around several tyrosine residues is in good agreement with the predicted optimal motif for the EGFR (Songyang et al., 1995). While this paper was in review, several other investigators reported that p120\(^{cd}\) associated with the activated EGFR and became tyrosine-phosphorylated in EGF-treated cells (Tanaka et al., 1995; Galisteo et al., 1995), and it was suggested that p120\(^{cd}\) binds both directly to the autophosphorylated C-terminal tail of the EGFR as well as indirectly through an unknown adaptor protein (Galisteo et al., 1995). p85 was also found to associate with p120\(^{cd}\) in activated T cells (Meisner et al., 1995; Hartley et al., 1995; Fukazawa et al., 1995), and in vitro binding data indicated that this association was mediated through both SH2 and SH3 domains of p85 (Hartley et al., 1995; Fukazawa et al., 1995).

As a result of our previous study (Soltoff et al., 1994), we concluded that there were at least two different adapter proteins involved in the activation of PI 3-kinase by EGF (Soltoff et al., 1994). We demonstrated that EGF stimulated the tyrosine phosphorylation of ErbB3 in A431 cells and that PI 3-kinase was recruited to the tyrosine-phosphorylated ErbB3 protein. Similar findings were also reported by Kim et al. (1994). Since this mechanism did not account for the activation of PI 3-kinase activity in PC12 or A549 cells, we concluded that a second mechanism must occur in these cells. The data presented in this paper indicate that p120\(^{cd}\) serves as an adapter protein for the EGF-dependent activation of PI 3-kinase in PC12 and A549 cells. The effects of EGF on these cells were distinct from other growth factors (notably insulin) that act via the stimulation of tyrosine kinases. In addition, the new results obtained using A431 cells indicate that two complementary systems (ErbB3 and p120\(^{cd}\)) involved in the EGF-dependent activation of PI 3-kinase are present in the same cell. It remains to be determined whether there are some cells that express ErbB3 and do not express p120\(^{cd}\).

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