Inhibition of Src family kinases blocks EGF-induced activation of Akt, phosphorylation of c-Cbl, and ubiquitination of the EGF receptor*

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Running Title: EGF-induced phosphorylation of c-Cbl requires Src-like kinases

Key words: Akt, Src, Cbl, EGF receptor, phosphatidylinositol 3-kinase, signal transduction, ubiquitin

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ABSTRACT

Stimulation of T47D cells with epidermal growth factor results in the activation of the receptor's intrinsic tyrosine kinases, the phosphorylation of multiple cellular proteins including the receptor, scaffold molecules such as c-Cbl, adapter molecules such as Shc, as well as the serine/threonine protein kinase Akt. We demonstrate that EGF stimulation of T47D cells results in the activation of the Src tyrosine protein kinase, and that the Src kinase inhibitor PP1 blocks the EGF-induced phosphorylation of c-Cbl, but not the activation/phosphorylation of the EGF receptor itself. PP1 also blocks EGF-induced ubiquitination of the EGF receptor, which is presumably mediated by phosphorylated c-Cbl. Src is associated with c-Cbl and we have previously demonstrated that the Src-like kinase Fyn can phosphorylate c-Cbl at a preferred binding site for the p85 subunit of phosphatidylinositol 3'-kinase. PP1 treatment blocks EGF-induced activation of the anti-apoptotic protein kinase Akt suggesting that Src may regulate activation of Akt, perhaps by a Src→c-Cbl→PI3-kinase→Akt pathway.
INTRODUCTION

Signal transduction by the EGF$^1$ receptor requires activation of the receptor's tyrosine kinase to activate downstream signaling molecules. Downstream signaling pathways activated by the EGF receptor include the classic Ras/Raf/MAP kinase pathways, phospholipase C, and PI3-kinase. Although some signaling molecules are activated by direct binding to the activated EGF receptor, mediated by the direct binding of their SH2 domains to phosphorylated tyrosine residues, other signaling molecules do not appear to bind directly to the receptor itself and yet they are activated in a receptor-dependent manner. With regard to the EGF receptor, examples of the former include phospholipase C, while an example of the latter type of interaction is PI3-kinase, which does not appear to bind to the EGF receptor. In recent years it has become clear that large scaffolding molecules such as IRS-1, Gab1, Gab2, and perhaps c-Cbl, may function in cooperation with growth factor receptors to regulate activation of downstream signaling molecules that do not bind directly to growth factor receptors$^{1-4}$. These molecules are all relatively large in size (Mr 95,000 to 130,000) and contain numerous tyrosine residues that become phosphorylated following receptor engagement. In our studies we have focused upon c-Cbl, a 120,000-dalton protein with 22 tyrosine residues.

c-Cbl is the first member of the Cbl family of scaffolding molecules which includes c-Cbl, Cbl-b, Cbl-3, D-Cbl from Drosophila, and the C. elegans homologue Sli-1$^{5,6}$. Engagement of numerous receptors results in the phosphorylation of c-Cbl; these receptors include the EGF receptor$^{7-9}$, the interleukin-3 receptor$^{10,11}$, the erythropoietin receptor$^{8,10}$, the prolactin receptor$^{12}$, integrins$^{13,14}$, the T-cell receptor$^{15,16}$, and the B-cell receptor$^{17,18}$. c-Cbl has been shown to be associated with numerous signaling
molecules (Src, Fyn, Lyn, Syk, ZAP70, and PI3-kinase)\textsuperscript{8,11,16,17,19}, as well as several adapter molecules (Shc, Crk, and Grb2)\textsuperscript{9,16,17,20-22}. The association of c-Cbl with PI3-kinase suggests that c-Cbl could function as a scaffolding molecule that regulates activation of downstream signaling molecules. c-Cbl can be phosphorylated by both Src-like kinases as well as members of the Syk/ZAP70 family of tyrosine kinases\textsuperscript{23-29}, however, it is not clear which specific tyrosine residues are phosphorylated by these kinases, and whether different receptors utilize different tyrosine kinases to phosphorylate different regions of c-Cbl.

Complicating the view of c-Cbl as merely a molecular scaffold is the observation that the \textit{C. elegans} homologue of c-Cbl, \textit{Sli-1}, functions as a negative regulator of the \textit{C. elegans} EGF receptor homologue \textit{let-63}\textsuperscript{5}. Consistent with this observation, overexpression of c-Cbl in fibroblasts resulted in the down-regulation of the EGF receptor\textsuperscript{30,31}. A mechanism explaining this observation was revealed when it was realized that the RING finger motif of c-Cbl could function as part of a ubiquitin ligase complex\textsuperscript{32-34}, and that overexpression of c-Cbl in fibroblasts stimulated the ubiquitination of the EGF receptor leading to its degradation\textsuperscript{33-36}. This leads to the question of whether c-Cbl’s primary cellular function is as a scaffolding molecule, a ubiquitin ligase, or both.

In this paper we demonstrate that EGF stimulated phosphorylation of c-Cbl appears to require a PP1-sensitive tyrosine kinase, presumably one of the members of the Src family of tyrosine kinases. Blocking phosphorylation of c-Cbl with PP1 also blocks ubiquitination of the EGF receptor following EGF stimulation, although the EGFR is activated and auto-phosphorylated. Furthermore, the activation of Src family members
regulates activation of the anti-apoptotic protein kinase Akt, perhaps by a Src→c-Cbl→PI3-kinase→Akt pathway.
MATERIALS AND METHODS

Cells and Cell culture. The human breast cancer cell line T47D was obtained from Dr. Dean Edwards (University of Colorado Health Sciences Center, Denver, CO). MCF12A cells were obtained from the University of Colorado Cancer Center Tissue Culture Core Facility. T47D cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1 mM L-glutamine, 1X nonessential amino acids, 0.4 μg/ml insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin. MCF12A were cultured in a 50:50 mixture of Dulbecco’s modified Eagle’s medium and Hamm’s F12 supplemented with 5% horse serum, 1 mM L-glutamine, 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.1 μg/ml cholera toxin, 20 ng/ml EGF, 100 U/ml penicillin, and 100 μg/ml streptomycin. Fetal calf serum was from Summit Biotechnology (Fort Collins, CO), horse serum from Sigma Chemical Company (St. Louis, MO), and charcoal stripped fetal calf serum was obtained from HyClone (Logan, UT). Murine epidermal growth factor was obtained from Collaborative Biomedical Products (Bedford, MA). All other media components were from Life Technologies (Gaithersburg, MD). Cells to be stimulated with EGF were cultured overnight (for 16 hours) in culture media lacking fetal calf serum prior to stimulation with growth factors at the indicated concentrations and for the times indicated prior to harvest. PP1 was obtained from CalBiochem (LaJolla, CA) or Alexis Biochemical Company (San Diego, CA).

Immunoprecipitation and Immunoblotting. Cells to be immunoprecipitated were lysed in EB (50 mM NaCl, 10 mM Tris, pH 7.4, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate with 100 U/ml aprotinin), and the lysates clarified by
spinning at 10,000 rpm in a Savant RCF13K refrigerated microfuge for 30 minutes. For 
the experiments involving ubiquitin, 10 mM NEM and 50 µM LLnL were added to the 
extraction buffer. 1-2 µg of the indicated antibodies were added to 1 ml cell lysate 
corresponding to 1 mg total cellular protein as determined by the Pierce BCA protein 
assay (Pierce Chemical Company, Rockford, IL), and placed on a rocking platform for 1 
hour at 4°C. The immune complexes were collected by adding 30 µl Pansorbin 
(Calbiochem) to each immunoprecipitate for 1 hour. The bound proteins were washed 
three times with lysis buffer and the immunoprecipitated proteins resolved by SDS 
polyacrylamide gel electrophoresis. The resolved proteins were electrotransferred to 
Immobilon membranes (Millipore, Bedford, MA). Detection of proteins by 
immunoblotting was conducted using the Enhanced Chemiluminescence Lighting (ECL) 
system according to the manufacturer’s recommendations (Amersham Corp., Arlington 
Heights, IL). In some experiments whole cell lysates were directly analyzed by 
immunoblotting without immunoprecipitation.

Agarose-conjugated anti-phosphotyrosine monoclonal antibody 4G10 (Catalogue 
# 16-101), and a rabbit polyclonal antibody to the p85 subunit of PI3-kinase (Catalogue # 
06-195) were obtained from Upstate Biotechnology, Inc (Lake Placid, NY). Anti-Shc 
(Catalogue # S14630), anti-EGF receptor antibody (Catalogue #E12020), and a second 
anti-p85 antibody (Catalogue # 65721A) were obtained from Transduction Laboratories 
(Lexington, KY). A polyclonal antibody directed against c-Cbl was obtained from Santa 
Cruz Biotechnology (Catalogue # sc-170, Santa Cruz, CA). EGFR antibodies used in the 
experiments in Figure 8 were also from Santa Cruz Biotechnology (Catalogue # sc-120
for immune precipitations and sc-03 for western blotting). Monoclonal antibody 4G10 directed against phosphotyrosine was kindly provided by Dr. Brian Druker (University of Oregon Health Sciences Center, Portland, OR). Anti-Src antibody 2-17 was produced in this laboratory as were two monoclonal antibodies directed against c-Cbl (802H6 for immunoprecipitation and A672E4 for immunoblotting). The anti-ubiquitin monoclonal antibody Ubi-1 was obtained from Zymed (Catalogue # 13-1600, South San Francisco, CA).

**Kinase assays.** PI kinase assay was conducted as described previously. The immune complex protein kinase assay for Src was conducted as previously described.

**GST-fusion proteins and Binding Assays.** A series of GST fusion proteins that include different regions of c-Cbl have been previously described. The names for the fusion proteins used in this study and the amino acids included in the proteins are as follows: GST-PRO (proline rich region, amino acids 475-694); GST-LZIP (the C-terminal end of c-Cbl which includes the leucine zipper region, amino acids 426-906, GST-LZIP-Y731F (GST-LZIP with tyrosine731 mutated to a phenylalanine). A cDNA clone of human c-Cbl was provided by W. Langdon (University of Western Australia, Perth, Australia) and was used as the template to make all of these GST fusion proteins. And all primers were taken directly from the published DNA sequence of this cDNA. Mutagenesis of individual codons was performed according to the manufacture’s recommendations using the Ex-Site mutagenesis kit from Stratagene (LaJolla, CA).
GST fusion proteins encoding the N- and C-terminal SH2 domains, and the SH3 domain of the p85 subunit of PI3-kinase were kindly provided by Dr. Lewis Cantley (Harvard Medical School, Boston, MA).

The purification of the GST fusion proteins was conducted as described previously \(^{11}\). Binding assays were conducted by adding 2 nmol of the desired GST-fusion protein to a cell lysate prepared in RIPA buffer as described above, in a final volume of 1 ml. Following a 1 hour incubation at 4°C on a rocking platform, 40 ul of glutathione agarose (Pharmacia Biotech, Piscataway, NJ) was added and incubated for 1 hour. The bound proteins were washed three times with RIPA, resolved on SDS polyacrylamide gels, and subjected to immunoblotting as described above.
RESULTS

EGF-induced phosphorylation of c-Cbl and association with PI3-kinase. EGF receptor family members are thought to contribute to the development of cancer in numerous epithelial tissues including the breast, lung, ovaries and uterus\textsuperscript{39-41}. Stimulation of cells with EGF has been reported to activate numerous signaling molecules, including the large adapter molecule c-Cbl\textsuperscript{8,20,42}. To examine the role of c-Cbl in the proliferation of human breast cancer cells, we first examined the effect of EGF stimulation upon c-Cbl phosphorylation in T47D cells. EGF-stimulation resulted in the rapid tyrosine phosphorylation of c-Cbl and its association with multiple tyrosine-phosphorylated proteins (Figure 1). A major tyrosine phosphorylated protein of approximately 185 kDa was observed to co-precipitate with c-Cbl, along with minor bands of 62 and 54 kDa (Figure 1). Based upon the work of other investigators, the major band of 185 kDa appears to be the EGF receptor\textsuperscript{7,8,20,42}. The minor bands of 62 and 54 kDa appear to correspond to tyrosine-phosphorylated Shc as indicated by reprobing the immunoblot with anti-Shc antibody (Figure 1, Panel B). The position and amount of c-Cbl in the anti-phosphotyrosine immunoblot was confirmed by reprobing the blot with anti-c-Cbl antibody. Although the amount of c-Cbl protein appears to decrease following EGF-stimulation (Figure 1C), this appears to be an artifact of the way this experiment was done. When a similar immunoblot was first probed with anti-Cbl antibody, there was no decrease in the amount of c-Cbl protein (data not shown), whereas when the blot was probed with anti-phosphotyrosine antibody first followed by the anti-Cbl antibody, it appeared that there was a transient decrease in the amount of c-Cbl protein (Figure 1C).
This suggests to us that the extensive signal detected with anti-phosphotyrosine antibody may prevent the binding of a portion of the anti-Cbl antibody.

Our previous work with the interleukin-3 and prolactin receptors indicated that there was an increase in c-Cbl-associated PI3-kinase activity following cytokine stimulation, even though we could not detect the phosphorylation of the p85 subunit of PI3-kinase. No readily apparent tyrosine-phosphorylated protein with a molecular weight corresponding to that of the p85 subunit of PI3-kinase was detected in EGF-stimulated T47D cells (Figure 1, panel A), however, we did detect the rapid and transient association of the p85 subunit of PI3-kinase with c-Cbl by immunoblotting with anti-p85 antibody (Figure 1, panel D, lanes 2 and 3). In the study shown in Figure 1, the amount of p85 associated with c-Cbl peaked quickly (2 minutes after stimulation with EGF in the study shown), and declined after that time. These data suggest that there is a rapid and transient association of c-Cbl with the EGF receptor, Shc, and the p85 subunit of PI3-kinase. Of these interactions, the complex of c-Cbl with Shc appears to be more stable.

A dose-response study was conducted to determine what concentration of EGF was required to induce phosphorylation of c-Cbl. T47D cells were stimulated with 0-200 ng/ml EGF for 10 minutes, the cells lysed, and immunoprecipitated with anti-Cbl antibody. The resolved proteins were immunoblotted with anti-phosphotyrosine antibody 4G10 (Figure 2). Treatment of T47D cells with EGF concentrations as low as 5 ng/ml was able to induce tyrosine phosphorylation of both c-Cbl and the EGF receptor (Figure 2). Stimulation with concentrations greater than 100 ng/ml caused no further increase in the phosphorylation of either c-Cbl or the EGF receptor. Stimulation of T47D cells also caused a dose-dependent increase in the phosphorylation of a protein with a molecular
weight corresponding to two different isoforms of Shc (Figure 2). There was no effect of EGF stimulation upon the amount of c-Cbl protein present in the immunoprecipitates (data not shown).

**Rapid transient association of c-Cbl and PI 3-kinase.** Studies by other investigators have suggested that the activation of PI3-kinase by growth factors is critical in the generation of either a proliferative signal by growth factor receptors and/or in the suppression of apoptosis \(^{43-47}\). The transformation of mammary epithelial cells into tumor cells may involve the induction of proliferative signals, anti-apoptotic signals, or perhaps both. For this reason we wished to further investigate the data shown in Figure 1 regarding the association of PI3-kinase with c-Cbl following stimulation of T47D cells with EGF. We first asked whether PI kinase activity was also associated with c-Cbl following EGF stimulation of T47D cells. T47D cells were stimulated with EGF for 0-20 minutes, the cells lysed and immunoprecipitated with anti-Cbl antibody. One tenth of the immunoprecipitated protein was used in a PI kinase assay and the remainder of the protein was subjected to immunoblotting with an anti-p85 monoclonal antibody (Figure 3A). Consistent with the results shown in Figure 1, the rapid phosphorylation of c-Cbl was noted, as was the co-immunoprecipitation of a 185 kDa protein corresponding to the EGF receptor (data not shown). The rapid association of the p85 subunit of PI3-kinase with c-Cbl following EGF stimulation was also detected (Figure 3A). The maximal amount of c-Cbl-associated p85 was detectable at 1-2 minutes and declined after that time (Figure 3A). It does appear that there are 2 bands in the anti-p85 immunoblot and we do not know whether this represents modification of p85, or the presence of different p85
isoforms. c-Cbl-associated PI kinase activity was also examined using a PI kinase assay. A maximal amount of PI kinase activity was also detected after one minute of stimulation with EGF (Figure 3B, lane 9). Although c-Cbl-associated PI kinase activity was still detectable 2 minutes post stimulation, it declined dramatically after that time (Figure 3B). Other studies have shown that c-Cbl-associated PI kinase activity can be detected after 5 minutes of stimulation with EGF, however, kinase activity was not detectable at later time points (10-30 minutes) (data not shown). Phosphorimager quantitation indicated that there is 2-5-fold more c-Cbl-associated PI kinase activity at one-minute post EGF stimulation than present 2 minutes after EGF stimulation. There does not appear to be as dramatic a difference in the amount of c-Cbl-associated p85 protein at 1 and 2 minutes post EGF-stimulation when compared to the difference in PI kinase activity observed at these two time points (compare Figures 3A and 3B), however, the kinase assay should reflect the amount of activated PI3-kinase, not the total amount of p85. These data indicated that both p85 and PI kinase activity are rapidly associated with c-Cbl following stimulation of T47D cells with EGF, and the kinetics of PI kinase activation and p85 binding to c-Cbl suggest that this event may represent a very early signaling event.

The binding of c-Cbl to p85 was examined in a series of binding assays utilizing GST fusion proteins that contained different regions of either p85 or c-Cbl. The p85 subunit of PI3-kinase contains two SH2 domains (referred to as the N-SH2 and C-SH2 for the N- and C-terminal SH2 domains, respectively), and a single SH3 domain. The ability of GST fusion proteins encoding these three domains to bind to c-Cbl present in lysates of T47D cells was investigated by adding 2 nmols of the indicated GST fusion proteins to lysates of unstimulated and EGF-stimulated T47D cells (Figure 4). The GST-
p85-SH3 domain was observed to bind to c-Cbl in lysates of both unstimulated and EGF-stimulated T47D cells (Figure 4, lanes 7 and 8). When lysates of EGF-stimulated T47D cells were used in the binding assay, there was a decrease in the amount of c-Cbl that bound to the GST-p85 SH3 domain fusion protein. We do not believe that this reflects a decrease in the amount of c-Cbl present in these cells at this time point since immunoblotting of these lysates with anti-Cbl antibody does not indicate such a decrease (data not shown). Instead we believe that the c-Cbl protein present in lysates of EGF stimulated T47D cells may be complexed to other proteins so as to prevent binding to the GST fusion protein. We did detect a small amount of c-Cbl bound to the GST-p85-N-SH2 fusion protein when lysates of EGF-stimulated T47D cells were used (Figure 4, lane 4), however, no binding of c-Cbl was detected to the GST-p85-C-SH2 domain fusion protein. The low amount of c-Cbl that bound to the SH2 domain fusion proteins could indicate that either high affinity phosphotyrosine-dependent binding requires both SH2 domains of p85, or that it requires additional sequences other than just the SH2 domain. Alternatively, tyrosine-phosphorylated c-Cbl present in the lysates of EGF-stimulated T47D cells is complexed with other signaling molecules that prevents the binding of these GST fusion proteins. Previous investigators did not detect the binding of single SH2 domains of p85 to c-Cbl.

To identify the region of c-Cbl to which p85 bound, a series of GST fusion proteins were prepared that encoded different regions of c-Cbl. Three large fusion proteins were used that encoded the tyrosine-rich region of c-Cbl (amino acids 228-479), the proline-rich region (amino acids 475-694), and a region we refer to as the leucine zipper region (amino acids 426-906). All of these fusion proteins were prepared in an
unphosphorylated and a tyrosine-phosphorylated form by expression in either DH5α or TKB1 bacteria respectively. The TKB1 strain of E. coli contains an inducible elk1 tyrosine kinase that readily phosphorylates bacterial fusion proteins expressed in the same cells on tyrosine residues. GST fusion proteins prepared in TKB1 bacteria were tyrosine-phosphorylated as indicated by anti-phosphotyrosine immunoblotting, while GST fusion proteins prepared in DH5α bacteria contained no detectable phosphotyrosine. No attempts were made to determine the relative stoichiometry with which different tyrosine residues were phosphorylated. Binding assays were conducted with unphosphorylated and tyrosine-phosphorylated GST fusion proteins representing different regions of c-Cbl. As shown in Figure 4B, no binding of p85 was observed to GST alone (see lane 9). A small amount of p85 was observed to bind to the tyrosine-phosphorylated GST-PRO-RICH region (Fig 4B, lane 11). The major binding site for p85 appears to be to a phosphorylated tyrosine residue in the “leucine zipper” region of c-Cbl as indicated by the extensive binding of p85 to the phosphorylated GST-LZIP fusion protein and the total absence of binding to the nonphosphorylated GST-LZIP protein (Figure 4B, lanes 12 versus 13). We observed no binding of p85 to the nonphosphorylated or tyrosine-phosphorylated GST-YRICH fusion protein (data not shown). There are five tyrosine residues present in the GST-LZIP fusion protein. The amino acids numbers and the sequence context for these different tyrosine residues are listed in Table 1. Only one of these seven tyrosine residues would be predicted to represent a binding site of the SH2 domain of the p85 subunit of PI3-kinase; tyrosine\textsuperscript{731} lies in the sequence YEAM that would be predicted to a binding site for p85 based upon the described consensus sequence of YXXM for the SH2 domains of p85\textsuperscript{49}. To determine whether tyrosine\textsuperscript{731} represented
the major binding site for p85 in this region of c-Cbl we used oligo-directed mutagenesis to mutate tyrosine\textsuperscript{731} to a phenylalanine. This mutated GST fusion protein referred to as GST-LZIP-Y731F was prepared in unphosphorylated and tyrosine-phosphorylated versions as described above. Anti-phosphotyrosine immunoblotting indicated that the phosphorylated GST-LZIP-Y731F fusion protein was still phosphorylated on one or more of the four remaining tyrosine residues. No binding of p85 was observed to the pGST-LZIP-Y731F fusion protein, suggesting the tyrosine\textsuperscript{731} in c-Cbl represents the major phosphotyrosine-dependent binding site for the p85 subunit of PI3-kinase (Figure 4B, lane 14).

To demonstrate that the binding of p85 observed in Figure 4B corresponded to PI kinase activity, one tenth of the protein that bound to the GST fusion proteins was used in a PI kinase assay to detect the present of this enzyme. As shown in Figure 4C, PI kinase activity was only detected in the protein bound to the tyrosine-phosphorylated GST-LZIP (pGST-LZIP) fusion protein (Figure 4C, lane 20). No activity was detected in binding reactions conducted with either the unphosphorylated or tyrosine-phosphorylated GST-LZIP-Y731F fusion proteins (Figure 4C, lanes 21 and 22). Although we did detect the binding of p85 to the phosphorylated GST-PRO-RICH fusion protein (Figure 4B, lane 11), we did not detect any PI kinase activity in this fraction (Figure 4C, lane 18), indicating that this protein must not be enzymatically active, or that there was not enough kinase activity to detect by this approach.

**Phosphorylation of c-Cbl occurs in a Src-kinase-dependent manner.** We have previously demonstrated that c-Cbl is constitutively associated with one or more members
of the Src-family of tyrosine kinases\textsuperscript{11,38}. Furthermore we demonstrated that Fyn, but not
Jak2, immunoprecipitated from prolactin-stimulated Nb2 cells could phosphorylate
 tyrosine\textsuperscript{731} in c-Cbl, the critical binding site for the p85 subunit of PI3-kinase\textsuperscript{38}. These
data suggest the interaction of Src-like kinases could be important in regulating PI3-
kinase activation by the EGF receptor. To investigate whether Src was activated by EGF
in T47D cells, unstimulated and EGF-stimulated cells were lysed and
immunoprecipitated with an anti-Src monoclonal antibody, and the immunoprecipitated
proteins used in an immune complex protein kinase assay. As can be seen in Figure 5,
EGF stimulation of T47D cells results in the activation of Src kinase as determined by the
autophosphorylation of Src (Figure 5A), as well as the phosphorylation of the exogenous
substrate enolase (data not shown). Unstimulated T47D cells appear to have a basal level
of Src kinase activity under these conditions, however activation of Src could be detected
by one minute and appeared to decline after five minutes (Figure 5A). There was no
change in the amount of the Src protein for at least 60 minutes after stimulation with EGF
(Figure 5B, and data not shown). Consistent results were obtained in four independent
studies. Activation of the Fyn tyrosine kinase was also observed (data not shown).
Consistent with our previous data, Src was observed to co-immunoprecipitate with c-Cbl
in unstimulated cells, although there appeared to be a transient decrease in the amount of
Src that co-precipitated with c-Cbl at 1 and 2 minutes after stimulation with EGF (Figure
5C and 5D).

To extend this observation, we have used the Src-family chemical inhibitor PP1 to
determine whether Src-family kinases are responsible for the EGF-induced
phosphorylation of c-Cbl. The crystal structure of the very similar compound PP2 bound
to the active site of HCK indicates that the specificity of PP1 and PP2 lies in the binding of a side chain methyl or chlorine, (depending upon whether it is PP1 or PP2), to a hydrophobic side-pocket in the active site of Src-like kinases. This site is not conserved in other tyrosine protein kinases. Pretreatment of T47D cells with PP1 resulted in a dramatic decrease in the phosphorylation of c-Cbl following EGF stimulation of T47D cells (Figure 6A). Densitometric measurements indicate that phosphorylation of c-Cbl was decreased by greater than 90% (data not shown). There was no decrease in the amount of the c-Cbl protein present in PP1-treated T47D cells compared to the amount of c-Cbl present in untreated T47D cells (Figure 6B).

PP1 has been described as an inhibitor specific for Src-family kinases, although PP1 has been reported to inhibit the EGF receptor tyrosine kinase at higher concentrations. To determine whether phosphorylation of the EGF receptor, largely due to autophosphorylation, was inhibited by PP1, anti-EGF receptor immunoprecipitates were immunoblotted with an anti-phosphotyrosine antibody (Figure 6C). The results of these studies indicate that PP1 did not significantly inhibit the phosphorylation of the EGF receptor following stimulation with EGF (Figure 6C). There was no effect of PP1 upon the amount of the EGF receptor present in these cells (Figure 6D). These data suggest that the effect of PP1 upon the phosphorylation of c-Cbl was not largely mediated by inhibition of the EGF receptor's intrinsic tyrosine kinase. To further extend this observation, we examined the effect of PP1 upon the pattern of tyrosine-phosphorylated proteins present in T47D cells following stimulation with EGF. As shown in Figure 6E, PP1 did not block the EGF-induced phosphorylation of the protein corresponding to the EGF receptor, however, the phosphorylation of other proteins was affected. There was a
significant effect of PP1 upon the phosphorylation of several proteins in the 120-150,000 Mr range, and this complex includes c-Cbl (Figure 6E). In addition the phosphorylation of a protein with a Mr of 65,000 was also significantly diminished by PP1 treatment (Figure 6E). It should be noted that this protein does not co-immunoprecipitate with c-Cbl (Figure 1), and we have not determined the identity of this protein. PP1 also diminished the phosphorylation of a protein with a Mr of 54,000 which appears to correspond to one of the isoforms of Shc (Figure 6E).

PP1 treatment of T47D cells also prevented co-immunoprecipitation of the tyrosine-phosphorylated EGF receptor with c-Cbl (Figure 6A). Since the EGF receptor is present and tyrosine phosphorylated (Figure 6C and D), this suggests that either the Src-dependent phosphorylation of c-Cbl is required for c-Cbl to bind to the EGF receptor, or that the putative tyrosine residue in the cytoplasmic tail of the EGF receptor to which c-Cbl binds is in fact phosphorylated by a Src-like kinase. We can not distinguish between these possibilities at this time.

**Activation of Akt occurs in a Src-kinase dependent manner.** Numerous studies have addressed the role of PI3-kinase in stimulating cell proliferation and, most recently, in suppressing apoptosis \(^{43-46}\). Inhibition of PI3-kinase induces apoptosis of cells even when they are cultured in the presence of survival/growth factors \(^{44-46,52}\). The discovery that PI3-kinase regulates the activation of the anti-apoptotic protein kinase Akt has provided a molecular basis for these observations \(^{43,44,46}\). Data presented above indicate that phosphorylation of c-Cbl occurs in a Src-kinase-dependent manner, and that phosphorylation of c-Cbl at tyrosine\(^ {731}\) could regulate activation of PI3-kinase through
binding of the p85 subunit to c-Cbl. If this is the case, then one would expect that inhibition of Src-like kinases might also block activation of the Akt protein kinase. To test this hypothesis T47D cells were treated with PP1 or solvent control then stimulated with EGF for 0-60 minutes, and the activation of Akt examined by immunoblotting with an anti-phospho-Akt antibody (Figure 7). The activation of Akt requires phosphorylation of both threonine$^{308}$ and serine$^{473}$, and comparable results are obtained by either immunoblotting with the anti-phospho-Akt (serine$^{473}$) antibody or by doing an immune protein complex protein kinase assay (data not shown). Treatment of T47D cells with PP1 blocked EGF-induced activation of Akt (Figure 7A), although a small amount of activated Akt could still be detected at the 30-minute time point. PP1 had no effect upon the level of Akt protein (Figure 7B) indicating that the decrease in the amount of activated Akt is not due to the loss of Akt protein. This data suggested that EGF-induced activation of Akt occurs in a Src-kinase-dependent manner and that activation/phosphorylation of the EGF receptor is not sufficient to drive Akt activation since this is not inhibited by PP1 (Figure 6).

EGF-induced ubiquitination of the EGF receptor occurs in a Src-kinase dependent manner. Studies by other investigators have indicated that tyrosine phosphorylation of c-Cbl is required for activation of its ubiquitin ligase activity following activation of the EGF receptor$^{36}$. Phosphorylated c-Cbl then ubiquitinates the EGF receptor, resulting in its down-regulation. The nature of the kinase responsible for the phosphorylation of c-Cbl has not been rigorously determined, but it has been proposed that the EGFR tyrosine kinase itself is responsible$^{36}$. However, in T47D cells, we find that tyrosine
phosphorylation of c-Cbl following EGF stimulation can be blocked by the Src-kinase inhibitor, PP1 (see Figure 6). We therefore asked whether PP1 can also prevent EGF-induced ubiquitination of the EGF receptor. In Figure 8, T47D cells were starved of growth factors overnight, and then stimulated with EGF. After exposure to EGF for the indicated times, cells were lysed into a buffer containing inhibitors of de-ubiquitinating enzymes and proteasomes. EGFR was then immunoprecipitated and analysed by western blotting with antibodies to EGFR (Figure 8A) or ubiquitin (Figure 8B). Under these conditions, EGF stimulation is seen to induce a transient alteration in the mobility of EGFR. Beginning at 1 minute after EGF stimulation, a lower mobility smear is evident above the major band of EGFR immunoreactivity (Figure 8A, compare lanes 1 and 2). This slowly migrating form of EGFR is increased at 5 minutes, and then diminishes at subsequent times. Probing parallel blots with anti-ubiquitin antibody reveals that the slowly migrating form of EGFR colocalizes with ubiquitin immunoreactivity (Figure 8B, lanes 2, 3 and 4). When T47D cells are pretreated with PP1 before EGF stimulation, no mobility shift in the EGF receptor is evident (Figure 8A, lanes 7 - 12), and ubiquitination of EGFR is largely abolished (Figure 8B, lanes 7 - 12). We next asked whether the ability of PP1 to block EGF-induced ubiquitination of the EGF receptor is somehow unique to T47D cells, or is more generalizable. Experiments with another human breast cell line, MCF12A, show results similar to T47D (Figure 8, C and D). In MCF12A cells the EGF-induced ubiquitination of EGFR appears to have a slightly longer time course, possibly due to the higher number of EGF receptors in these cells, but again, pretreatment with PP1 is seen to abolish EGF-induced EGFR ubiquitination. The concentration of PP1 used in these experiments does not block EGF-induced tyrosine phosphorylation of EGFR in
either cell line (Figure 6, and data not shown). These data suggest that activation of the
ubiquitin ligase activity of c-Cbl by tyrosine phosphorylation is mediated by a Src-like
kinase, rather than by the EGF receptor directly.
DISCUSSION

The activation of growth factor receptors leads to the activation of downstream signaling pathways in which one chemical signal is changed to other chemical signals. For example, activation of tyrosine kinases leads to activation of serine/threonine kinases, lipid kinases, phospholipases, as well as the induction of calcium fluxes. The activation of secondary signaling molecules is largely mediated through the binding of interaction motifs present in the secondary signaling molecules with activated receptors: most notably in this context is the SH2 domain that is present in many signaling molecules and which binds to phosphorylated tyrosine residues in a sequence-specific context. The binding of SH2-domain containing proteins to activated growth factor receptors either leads to the activation of these molecules following their phosphorylation, or results in their translocation to a new intracellular location where they are able to interact with substrates. In addition to the binding of secondary signaling molecules directly to activated receptors, secondary signaling molecules can also bind to scaffolding molecules that interact with receptors and appear to function as an extension of the receptor. Perhaps the most notable example of this type of molecule is IRS-1 that functions in coordination with the receptors for either insulin or insulin-like growth factor-1. These scaffolding molecules function as obligate partners for these growth factor receptors. There are several other large proteins with numerous tyrosine residues that could likely function as scaffolding molecules in an identical manner. These molecules include Gab-1, Gab-2, c-Cbl, Cbl-b, Cbl-3 and other related proteins. The critical question is what signaling pathways these scaffolding molecules regulate and whether redundant
regulatory pathways exist. Studies on Gab1 suggest that it may function to couple cytokine receptors to PI3-kinase\textsuperscript{54}, however, this may vary with the receptor examined, the cell type examined, and what other scaffolding molecules are present in a cell.

In this study we have demonstrated that the phosphorylation of c-Cbl following EGF stimulation of T47D cells appears to occur in a Src-like kinase dependent manner since treatment of cells with the Src inhibitor PP1 blocked phosphorylation of c-Cbl but not of the EGF receptor. We demonstrate that EGF stimulation resulted in the rapid association of PI3-kinase with c-Cbl in T47D cells. We have also demonstrated the direct binding of the p85 subunit of PI3-kinase to tyrosine\textsuperscript{731} in c-Cbl, however, it is possible that it also binds to other adapter or scaffolding molecules in vivo. In previous studies we have demonstrated that tyrosine\textsuperscript{731} is phosphorylated by the Src-like kinase Fyn, but not another tyrosine kinase Jak2\textsuperscript{38}. In this study we suggest that Src or perhaps Fyn, but not the EGF receptor may be the major kinase that phosphorylates c-Cbl in EGF-stimulated T47D cells. We also demonstrated that PP1 blocks the activation of Akt, another protein kinase which lies downstream of PI3-kinase. We have suggested that this may occur by a \textit{Src/Fyn→c-Cbl→PI3-kinase→Akt} pathway, although we can not rule occur the possibility that Src substrates other than c-Cbl could also regulate Akt activation. It is also possible that another PP1-sensitive kinase other than a Src-like kinase could regulate c-Cbl and/or Akt.

We have noticed, as have other investigators\textsuperscript{7,9,20}, that c-Cbl binds to the phosphorylated EGF receptor. Our data indicate however, that this interaction does not occur in PP1-treated cells even though the EGF receptor is phosphorylated on tyrosine residues. This suggests that either the phosphorylation of c-Cbl is required to allow it to
bind to the EGF receptor, or that the binding site for c-Cbl in the cytoplasmic tail of the EGF receptor is phosphorylated by a Src-like kinase. Recent studies have suggested that binding of c-Cbl to tyrosine£1045 in the cytoplasmic tail of the EGF receptor is required for ubiquitination and down-regulation of the EGF receptor £36. It has been assumed that this tyrosine residue is phosphorylated in an autocatalytic manner by the EGF receptor itself, although that has not been proven. Our data instead support the idea that a PP1-sensitive Src-like kinase phosphorylates the critical tyrosine residue in EGFR required for c-Cbl binding.

While the data presented here do not rule out the alternative hypothesis, that phosphorylation of c-Cbl is required for binding to the EGF receptor, we do not favor it. Binding studies of GST-Cbl fusion proteins with EGFR have not revealed a requirement for c-Cbl phosphorylation (Kassenbrock and Anderson, unpublished). In this context it is interesting to note that other investigators have suggested that phosphorylation of c-Cbl may be important in regulating its ubiquitin ligase activity £36. Mutation of tyrosine£371 in c-Cbl has been shown to inhibit the ubiquitin ligase function of c-Cbl, and based on such mutants, phosphorylation of tyrosine£371 has been proposed to be required for ubiquitin ligase activity £36. However, this interpretation has become controversial following structural studies suggesting that the sidechain of tyrosine£371 is buried and not solvent-accessible £55. This suggests that the critical phosphorylation site for ubiquitin ligase activation in c-Cbl may still remain to be identified, or that significant conformational changes in c-Cbl occur upon phosphorylation.

Our studies with PP1 suggest that regardless of which tyrosine residue is critical in regulating the ubiquitin ligase activity of c-Cbl, it is most likely phosphorylated by a
Src family member. We show here that the EGF-induced ubiquitination of the EGF receptor appears to be Src-kinase dependent since it is blocked in PP1-treated cells. This finding is surprising, since previous models had proposed that the EGF receptor directly phosphorylates c-Cbl, leading to activation of ubiquitin ligase activity and subsequent ubiquitination of EGFR. However, such a direct pathway should not be sensitive to PP1 at the concentrations employed.

EGFR and Src can interact bidirectionally. In addition to the activation of Src by EGFR observed following EGF stimulation, activation of Src by a variety of stimuli can result in ligand-independent activation of EGFR through Src-mediated tyrosine phosphorylation of EGFR (reviewed in 56), and biological synergy between the two tyrosine kinases can be observed 57.

Our model that a Src-like kinase is required for activating c-Cbl’s ubiquitin ligase activity, is consistent with recent findings from other laboratories. Yokouchi et al have recently suggested that Src-catalyzed phosphorylation of c-Cbl can activate c-Cbl’s ubiquitin ligase activity and lead to the subsequent ubiquitination of both c-Cbl and Src 58. And in a related system, Src family kinases were found to cooperate with c-Cbl in down-regulating the PDGF receptor α following PDGF-AA stimulation 59. We have not examined the ubiquitination of Src in our system at this time.

Our conclusions about the role of Src family kinases in the processes described here rely significantly on the use of the chemical inhibitor, PP1. PP1 is considered to be a specific inhibitor of Src family kinases 51. Determination of the crystal structure of Hck bound to PP2, a closely related compound in which the methyl group is replaced with a chlorine group, indicates that the specificity of PP2 and PP1 lie in the binding of the
methyl/chlorine group to a hydrophobic pocket which is not present in other kinases studied to date.\textsuperscript{50} PP1 and/or PP2 have been extensively used to examine the role of Src family members in signaling pathways including those activated by colony-stimulating factor-1\textsuperscript{60}, TRANCE\textsuperscript{61}, vascular endothelial growth factor\textsuperscript{62}, and platelet-derived growth factor\textsuperscript{63}. These studies indicate that these drugs can be used with some confidence.

Recently there has been great interest in Cbl family members following the demonstration that c-Cbl possesses ubiquitin ligase activity, and that overexpression of c-Cbl in cells results in the ubiquitination of the EGF receptor and its subsequent downregulation\textsuperscript{33,34,36}. It has been suggested that the phosphotyrosine binding domain of c-Cbl may be important in the selection of the proteins subjected to ubiquitin modification\textsuperscript{33,34,36}. These studies have utilized in vitro ubiquitination reactions to demonstrate the ability of the RING finger domain of c-Cbl to participate in the ubiquitin transfer reaction\textsuperscript{32}. The studies demonstrating that c-Cbl can stimulate the modification and downregulation of the EGF receptor have required overexpression of c-Cbl in these cells\textsuperscript{33,34,36}. Therefore it has not been clear whether under normal physiological conditions and normal concentrations of c-Cbl, if c-Cbl acts solely to down-regulate the EGF receptor, or if it has other functions such as the hypothesized role as a scaffolding molecule. The phenotype of c-Cbl knockout mice supports the idea that c-Cbl suppresses growth factor receptor function, however, other roles may not have been revealed in these mice, perhaps due to functional redundancy among other Cbl family members\textsuperscript{64}. We propose that under normal physiological conditions, c-Cbl may have multiple roles that include serving as a scaffold molecule to regulate downstream signaling molecules, and
also to control receptor levels and trafficking in the cell. We suggest that these disparate functions of c-Cbl may be separated in part in a temporal manner. Phosphorylation of c-Cbl may initially promote the formation of multi-protein signaling complexes, and then subsequently terminate signaling by ubiquitination of cell surface receptors and/or cytoplasmic signaling molecules. This model would also be consistent with recent data indicating that c-Cbl and Cbl-b are able to ubiquitinate Src, Syk, and PI3-kinase.

The disparate functions may be controlled by the sites that are phosphorylated on c-Cbl, the secondary signaling molecules that are associated with c-Cbl, and the cytosolic location of c-Cbl-EGF receptor signaling complexes. For example, the phosphorylation of a tyrosine residue in the amino terminal half of c-Cbl may be required for ubiquitin ligase activity, whereas PI3-kinase is regulated by a C-terminal phosphorylation event. Alternatively, access to the RING-finger motif might be blocked by other signaling molecules. Finally, the presence of c-Cbl/EGF receptor complexes in lipid rafts or other compartments might determine whether that complex is targeted for degradation, or remains active as a signaling complex. Therefore c-Cbl may act in multiple manners to enable it to regulate PI3-kinase and Akt, as well as mediating receptor downregulation.
FOOTNOTES

* This research was supported by grants from the National Institutes of Health: DK48879 and GM55754 to SMA, and DK48845 to GLJ.

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1 The abbreviations used in these manuscript are CSS, charcoal-stripped serum; EGF, epidermal growth factor; GST: glutathione S-transferase; IRS-1: insulin-regulated substrate-1; PI: phosphatidylinositol; PI3-kinase, phosphatidylinositol-3’kinase.; SH2 src-homology 2; SH3: src homology 3

ACKNOWLEDGMENTS

The authors thank Drs. Kathryn Schwertfeger and Mary Reyland for their comments on the manuscript.
FIGURE LEGENDS

Figure 1. EGF stimulation of T47D cells results in the phosphorylation of c-Cbl and its association with multiple signaling molecules. Confluent monolayers of T47D cells were cultured overnight in media lacking fetal calf serum to reduce the background level of tyrosine-phosphorylated proteins. The cells were stimulated with 100 ng/ml EGF for 0-120 minutes. The cells were lysed, the lysates clarified by centrifugation for 30 minutes, and then immunoprecipitated with anti-Cbl antibody. The immunoprecipitated proteins were resolved on a 7.5% SDS polyacrylamide gel and electrotransferred to an Immobilon membrane. In (A) the membrane was probed with anti-phosphotyrosine monoclonal antibody 4G10. The filter was then stripped and reprobed with (B) anti Shc antibody, (C) anti-Cbl antibody, and (D) anti-p85 antibody. The position of prestained protein markers are indicated on the left side of the top panel, and the position of proteins of interest are indicated by the arrowheads on the right side of each panel. The time in minutes of EGF stimulation is shown at the top of each lane, and the lane numbers are indicated at the bottom of each lane.

Figure 2. Dose response of EGF upon c-Cbl phosphorylation. T47D cells were cultured overnight in media lacking fetal calf serum to reduce the background level of tyrosine-phosphorylated proteins. The cells were stimulated with 0-200 ng/ml EGF for 10 minutes. The cells were then processed as described in Figure 1, immunoprecipitated with anti-Cbl antibody, and the immunoprecipitated proteins were resolved on a 7.5% SDS polyacrylamide gel. The immunoblot was probed with anti-phosphotyrosine
monoclonal antibody 4G10. The position of prestained protein markers are indicated on
the left side of the panel, and the position of proteins of interest are indicated by the
arrowheads on the right side of the panel. The concentration of EGF used to stimulate the
T47D cells is indicated at the top of each lane, and the lane numbers are indicated at the
bottom of each lane.

**Figure 3. Rapid association of the p85 subunit of PI3-kinase with c-Cbl following
stimulation of T47D cells with EGF.** T47D cells were cultured overnight in the absence
of fetal calf serum, then stimulated with 100 ng/ml EGF for 0-20 minutes (lanes 1-6).
The cells were then lysed, clarified by centrifugation, and 1 mg of the cell lysate was
immunoprecipitated with an anti-Cbl polyclonal antibody. As a control, a lysate of
unstimulated T47D cells was immunoprecipitated with a polyclonal antibody directed
against the p85 subunit of PI3-kinase (lane 7). **In panel A,** 90% of the
immunoprecipitated protein were resolved by SDS gel electrophoresis, and
immunoblotted with a monoclonal antibody directed against the p85 subunit of PI3-
kinase. The position of p85 is noted by the arrowhead on the right side of the panel. **In
panel B,** the remaining protein was used in a PI kinase assay as described in the Materials
and Methods section. The arrowheads on the right side of the panel indicate the positions
of the origin and of PIP. The time in minutes of EGF stimulation is shown at the top of
each lane, and the lane numbers are indicated at the bottom of each lane.

**Figure 4. Tyrosine731 in c-Cbl appears to be the major binding site for the p85
subunit of PI3-kinase.** In **panel A,** lysates of T47D cells were prepared and used in
binding assays with GST (lanes 1 and 2), GST-p85 N-SH2 (lanes 3 and 4), GST-p85 C-SH2 (lanes 5 and 6), or GST-p85 SH3 (lanes 7 and 8). The cell lysates were prepared from either unstimulated cells, (odd numbered lanes, indicated by - ), or following EGF stimulation (even numbered lanes, indicated by + ). The bound proteins were analyzed by immunoblotting with anti-Cbl antiserum to determine which regions of p85 were capable of binding to c-Cbl. The position of c-Cbl is indicated by the arrowhead on the right side of the panel. In panel B, lysates of unstimulated T47D cells were used in binding assays with unphosphorylated or tyrosine-phosphorylated fusion proteins representing different regions of c-Cbl. Lane 9, GST; lane 10, GST-PRO; lane 11, tyrosine-phosphorylated GST-PRO; lane 12, GST-LZIP; lane 13, tyrosine-phosphorylated GST-LZIP; lane 14, GST-LZIP-Y731F; and lane 15, tyrosine-phosphorylated GST-LZIP-Y731F. 90% of the protein bound to these fusion proteins was used in an anti-p85 immunoblot to identify the fusion proteins to which PI3-kinase was capable of binding. The arrowhead on the right side of the panel notes the position of the p85 subunit of PI3-kinase. In panel C, 10% of the protein bound to the GST c-Cbl fusion proteins in panel 4B above was used in a PI kinase assay to determine which fractions contained PI kinase activity. Lane 16, GST; lane 17, GST-PRO; lane 18, tyrosine-phosphorylated GST-PRO; lane 19, GST-LZIP; lane 20, tyrosine-phosphorylated GST-LZIP; lane 21, GST-LZIP-Y731F; and lane 22, tyrosine-phosphorylated GST-LZIP-Y731F. The positions of the origin and of PIP are noted by the arrowheads on the right side of the panel.

**Figure 5. EGF stimulates the activation of the Src tyrosine kinase.** Confluent monolayers of T47D cells were treated as described in Figure 1, then stimulated with 100
ng/ml EGF for 0-10 minutes (lanes 1-5). The cells were then lysed, the lysates clarified by centrifugation for 30 minutes, and 1 mg of the lysate immunoprecipitated with anti-Src monoclonal antibody 2-17 in a final volume of 1 ml. A). The immune complex protein kinase assay was conducted as described \(^{37}\) to determine the activation of Src by EGF. In B) a second set of cell lysates was immunoprecipitated with anti-Src antibody 2-17, and then immunoblotted with the same antibody to demonstrate the amount of Src in the immunoprecipitates. In C) a third set of cell lysates were immunoprecipitated with anti-Cbl antibody, the immunoprecipitated proteins resolved by SDS gel electrophoresis, and then immunoblotted with anti-Src antibody 2-17. In D) the filter in panel C was reprobed with a monoclonal antibody directed against c-Cbl. The time in minutes of EGF stimulation is shown at the top of each lane, and lane numbers are indicated at the bottom.

Figure 6. PP1 treatment inhibits EGF-induced phosphorylation of c-Cbl. Confluent monolayers of T47D cells were cultured overnight in the absence of fetal calf serum, and then either left untreated (lanes 1-6) or pretreated with 50 uM PP1 for 30 minutes (lanes 7-12). Both set of cells were then stimulated with 100 ng/ml EGF for 0-15 minutes, as indicated at the top of the figure. The cells were then lysed, and the lysates clarified by centrifugation. In A and B) 1 mg of the lysate was immunoprecipitated with anti-Cbl polyclonal antibody, the immunoprecipitated proteins resolved by SDS gel electrophoresis on a 7.5% gel, and immunoblotted with either anti-phosphotyrosine monoclonal antibody 4G10 (Panel A), or an anti-Cbl polyclonal antibody to demonstrate equal loading of the gel (Panel B). In C and D) 1 mg of cell lysate was
immunoprecipitated with anti-EGF receptor polyclonal antibody, the immunoprecipitated proteins resolved by SDS gel electrophoresis on a 7.5% gel, and immunoblotted with either anti-phosphotyrosine monoclonal antibody 4G10 (Panel C), or an anti-EGF receptor antibody to demonstrate equal loading of the gel and the migration pattern of the phosphorylated EGF receptor under both conditions (Panel D). In E) 1 mg of cell lysate was immunoprecipitated with agarose conjugated anti-phosphotyrosine monoclonal antibody 4G10, the immunoprecipitated proteins resolved by SDS gel electrophoresis on an 8% gel, and the filter immunobotted with anti-phosphotyrosine monoclonal antibody 4G10. In panel A the positions of the proteins of interest are indicated by bars on the right. In panel E, the positions of prestained molecular weight markers are on indicated on the right side of the panel.

**Figure 7. PP1 treatment inhibits EGF-induced activation of Akt.** Confluent monolayers of T47D cells were cultured overnight in the absence of fetal calf serum, and then either left untreated (lanes 1-5) or treated with 50 μM PP1 for 30 minutes (lanes 6-10). Both set of cells were then stimulated with 100 ng/ml EGF for 0-60 minutes. The cells were then lysed, and the lysates clarified by centrifugation. 50 μg total cellular protein was resolved on an 8% SDS polyacrylamide gel and the immunoblot probed with anti-phospho-Akt antibody (Panel A) or with anti-Akt antibody B). The position of the phospho-Akt and Akt are indicated on the right side of each panel. The time in minutes of EGF stimulation is shown at the top of the figure.
Figure 8. **PP1 treatment inhibits EGF-induced ubiquitination of the EGF receptor.**

**In A and B,** confluent monolayers of T47D cells were cultured overnight in the absence of fetal calf serum, and stimulated with 100 ng/ml EGF for 0, 1, 5, 10, 20, and 30 minutes, as indicated at the top of the figure. Lane numbers are indicated at the bottom of each panel. Cells corresponding to lanes 7 – 12 were pretreated with 50 μM PP1 30 minutes before stimulation. The cells were then lysed, EGFR was immunoprecipitated, and aliquots of the immunoprecipitates were resolved by SDS gel electrophoresis on duplicate 6 % gels followed by transfer to PVDF membranes for immunoblotting. The bars at the left of each panel indicate the position of a 208 kDa molecular weight marker.

**In A)** EGFR immunoprecipitates were immunoblotted with an anti-EGF receptor antibody to demonstrate the altered migration pattern of the EGF receptor after stimulation. **In B)** a parallel immunoblot of EGFR immunoprecipitates was probed with an anti-ubiquitin monoclonal antibody. **In C and D,** the same experiment was repeated with MCF12A cells.
REFERENCES


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Figure 5

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IP: Src, Kinase Assay

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IP: Cbl, Blot: Src

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IP: Cbl, Blot: Cbl
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IP: EGFR
Blot: EGFR

**B**

IP: EGFR
Blot: Ubiquitin

**C**

IP: EGFR
Blot: EGFR

**D**

IP: EGFR
Blot: Ubiquitin
Inhibition of Src family kinases blocks EGF-induced activation of Akt, phosphorylation of c-Cbl, and ubiquitination of the EGF receptor
C. Kenneth Kassenbrock, Seija Hunter, Pamela Garl, Gary L. Johnson and Steven M. Anderson

*J. Biol. Chem.* published online May 6, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201026200

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