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Abi-1 forms an epidermal growth factor-inducible complex with Cbl: role in receptor endocytosis

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Abstract

The Abl-interactor (Abi) proteins are involved in the regulation of actin polymerization and have recently been shown to modulate epidermal growth factor receptor (EGFR) endocytosis. Here we describe the identification of a novel complex between Abi-1 and the Cbl ubiquitin ligase that is induced by stimulation with EGF. Notably, an Abi-1 mutant lacking the SH3 domain (Δ SH3) fails to interact with Cbl and inhibits EGFR internalization. We show that expression of the Abi-1 Δ SH3 mutant inhibits Cbl accumulation at the plasma membrane after EGF treatment. We have previously shown that the oncogenic Abl tyrosine kinase inhibits EGFR internalization. Here we report that the oncogenic Abl kinase disrupts the EGF-inducible Abi-1/Cbl complex, highlighting the importance of Abl kinases and downstream effectors in the regulation of EGFR internalization. Thus, our work reveals a new role for oncogenic Abl tyrosine kinases in the regulation of the Abi-1/Cbl protein complex and uncovers a role for the Abi-1/Cbl complex in the regulation of EGFR endocytosis.

Keywords

Abi-1; Cbl; EGF receptor; endocytosis; Abl kinase

1 Introduction

EGFR internalization is mediated by large protein complexes that promote the movement of activated receptors away from the plasma membrane via clathrin-dependent and independent pathways [1–3]. Adaptor proteins such as Grb2 [4,5], CIN85 [6,7] and Eps15 [8] have well-established roles in EGFR internalization. The Cbl proteins can function both as ubiquitin ligases and as adaptor molecules during growth factor receptor internalization [9]. Moreover, Cbl proteins translocate *in vivo* to ligand-bound receptor tyrosine kinases (RTKs) and form ternary complexes with other endocytic regulators such as endophilin and CIN85 [10,11]. We have recently shown that activated Abl kinases play a negative role in the regulation of EGFR endocytosis [12]. Here, we examine the role of the Abl target, Abi-1, in this process. Abi-1 has been recently reported to modulate EGFR endocytosis through an interaction with N-WASP [13]. Here we uncover a novel interaction between Abi-1 and the Cbl ubiquitin ligase and show that Abi-1 mediates Cbl accumulation to the plasma membrane upon stimulation by EGF. Thus, Abi-1 mediated effects on EGFR endocytosis depend in part on its interaction with Cbl.

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The Abi adaptor proteins, Abi-1 and Abi-2, were initially identified as binding proteins and substrates of the Abl family of tyrosine kinases [14,15]. Subsequently, Abi proteins were shown to form part of a complex containing Wave 1 and 2, Nap-1 and Sra-1, which functions downstream of Rac-1 to promote lamellipodia formation and cytoskeletal reorganization in cells with activated growth factor receptors [16]. Abi proteins have been implicated in cell growth and transformation [14,15,17]. Additionally, Abi-1 has been shown to transduce signals from Ras to Rac and to play a role in cytoskeletal reorganization downstream of growth factor stimulation [18]. Thus, Abi proteins are linked to receptor and non-receptor tyrosine kinases, as well as to GTPase-mediated signaling events. We have shown that Abi proteins localize to sites of actin polymerization at the tips of lamellipodia and filopodia [19] and at sites of cell-cell adhesion [20]. More recently, our laboratory showed that Abi proteins play a critical role in the polymerization of actin at the immunological synapse following T cell receptor stimulation [21].

Initial suggestions for a role of Abi-1 in membrane trafficking came from the finding that Abi-1 interacts with Dynamin and Synaptojanin [22], which are involved in endocytosis [23,24]. Additionally, Abi-1 was reported to associate with macropinocytic vesicles and Abi-1 overexpression was shown to inhibit macropinocytosis [25]. More recently, downregulation of Abi-1 was shown to inhibit EGFR internalization, and this effect might be mediated by the interaction of Abi-1 with N-WASP [13].

Here we report a novel complex between Cbl and Abi-1, which is formed upon stimulation by EGF. Endogenous Abi-1 forms a complex with endogenous Cbl in COS7 cells only after EGF stimulation. Analysis of the Cbl/Abi-1 interaction indicates that this interaction is mediated by the Abi-1 SH3 domain, and that expression of an Abi-1 Δ SH3 mutant inhibits EGFR internalization. Moreover, we found that co-expression of wild type Cbl and Abi-1 promotes Abi-1 ubiquitination and that Cbl mutants with defective E-3 ligase activity failed to interact with Abi-1. Additionally, we report that an Abi-1 Δ SH3 mutant that fails to interact with Cbl inhibits Cbl accumulation to the plasma membrane in response to EGF. Therefore, Abi-1 is involved in targeting Cbl to the plasma membrane after EGF stimulation, and Abi-1 regulates EGFR internalization in part via a Cbl-dependent pathway.

2. Materials and Methods

2.1. Cell Culture

COS7 and 293T cells were obtained from ATCC and were grown in DMEM (Invitrogen Life Technologies) containing 10% FBS (Invitrogen). Additionally, Optimem I, Reduced Serum Media from Invitrogen was used to maintain the cells during Lipofectamine transfections.

2.2. Antibodies and Reagents

EGF was purchased from Calbiochem. The anti-Abi-2 (5421) and anti-Abi-1 (6987/6988) rabbit polyclonal antibodies were generated in our laboratory [26]. GFP-tagged Abi-1 was detected with a GFP polyclonal antibody (Clontech) for immunoprecipitations and a GFP-monoclonal antibody (Roche) for western blots. For Abl detection, we utilized Ab-3 mouse monoclonal against the Abl C-terminus (Oncogene). Cbl mouse monoclonal antibody clone 7G10 was from Upstate Biotechnology. To detect the EGFR, we employed an anti-EGFR antibody rabbit polyclonal antibody 1005, sc-03 (Santa Cruz Biotechnology), and monoclonal antibody 13G8 (Alexis Biochemicals). Tyrosine phosphorylated proteins were detected with a combination of mouse monoclonal antibodies, 4G10 (Upstate Biotechnology) and PY99 sc-7020 (Santa Cruz Biotechnology). HA-tagged Cbl was detected with a mouse monoclonal F-7 HA-probe (Santa Cruz Biotechnology). Tubulin antibody was purchased from SIGMA

(T6074). Anti-Ubiquitin P4D1 antibody was from Santa Cruz Biotechnology (sc-8017). EGF-Rhodamine was purchased from Molecular Probes.

2.3. DNA constructs and Transfections

pEYFP-C1, pEGFP-C1, and pEGFP-N3 were purchased from Clontech. EYFP-Abi-1 WT, EGFP-Abi-1 Δ SH3 and other Abi-1 mutants utilized were previously described [19]. pCMV2 Abi-1 WT and the mutant pCMV2-Abi-1 Δ SH3 were generated in our laboratory [27]. Abi-1 WT was cloned into pLEGFP C1 by digestion with Bgl II. Kinase-active (Abl-PP, [28]) and kinase-defective (Abl-KM) sequences were cloned into pCDNA3 for transfection experiments, or into MIGR1 for retroviral transduction experiments as previously described [29–31]. pXJ40-Cbl-WT and Cbl-mutants were generous gifts from Dr. Graeme R. Guy and pCDNA3 Cbl 381A and 70ZCbl were kindly provided by Dr. Yosef Yarden. Transfection of COS7 cells was carried out with Lipofectamine and Lipofectamine 2000 (Invitrogen). 293T cells were transfected with the Calcium Phosphate precipitation method.

2.4. Immunostaining

Cells were permeabilized in 4% Paraformaldehyde (Sigma), washed three times with 1X PBS, then blocked in 10 % Normal Donkey Serum before addition of primary antibodies. Abi-1 was detected by immunostaining with 5421 polyclonal antibody for endogenous Abi-1, and anti Flag monoclonal antibody for Flag-Abi-1. Cbl was detected with a mouse monoclonal antibody from Upstate Biotechnology. Secondary antibodies were from Jackson ImmunoResearch. Additionally, we carried out negative controls utilizing secondary antibody alone.

2.5. EGF-Rhodamine Internalization Assays

COS7 cells were plated in coverslips, transfected and serum-starved one day after transfection for 24 hours in DMEM containing 0.1% FBS. Cells were placed ice-cold binding medium (20 mM HEPES-NaOH pH 7.5, 130 mM NaCl, 0.1 % bovine serum albumin (BSA) for 30 minutes and treated with 40 ng/ml Rhodamine EGF (Molecular Probes, Eugene, Oregon) in serum-free DMEM for 1 h at 4 °C. To examine the internalization of EGF, the cells were incubated at 37 °C in EGF-free DMEM, followed by addition of acidic buffer as described in [12]. Cells were permeabilized in 0.5% Triton X-100 and immunostained for Abl. EGFR internalization was analyzed with a confocal microscope. Transfected proteins were detected with FITC, and Rhodamine-EGF complexes were detected with a CY3 filter channel and 3D-projections of slices throughout the cell. Cell counting was performed with the identity of the sample remaining unknown to the observer.

2.6. Cell lysis and immunoprecipitations

For co-immunoprecipitations we utilized 1 % Triton-X buffer (50 mM Hepes pH 7, 150 mM NaCl, 10 % Glycerol, 1% Triton-X-100, 1.5 mM MgCl₂, 1mM EGTA) with the addition of inhibitors (1 mM sodium orthovanadate, 1 mM PMSF, 25 mM sodium fluoride, 2 mM sodium pyrophosphate and 1 ug/ml aprotinin, leupeptin and pepstatin) and N-Ethylmaleimide (10 mM). Lysates were rotated for 20 min at 4 °C prior to centrifugation for 10 min at 14,300 rpm at 4 °C. After centrifugation, the supernatants were utilized for immunoprecipitations, incubated with primary antibodies for 2 hours and protein A or protein G-sepharose beads (Amersham) for two hours. Immunoprecipitates were washed three times with lysis buffer and bound complexes were resuspended in 2X SDS-sample buffer for analysis by SDS-PAGE.

2.7. Subcellular Fractionation

COS7 cells were subjected to EGF-stimulation for the indicated times 48 hours after transfection and 24 hours after serum-starvation. Cells were washed twice with ice-cold 1 X PBS and scraped in Hypotonic Buffer C (10 mM Hepes-K pH 7.6, 1.5 mM MgCl₂, 10 mM

KCl, 0.5 mM DTT, 1 mM NaEDTA, 1 mM NaEGTA), which was supplemented with inhibitors as described in [12]. Resuspended cells were placed at -80°C for 30 min and thawed in order to break down the membrane fraction. Cell suspensions were spun at 14,300 rpm for 30 min in a microcentrifuge at 4°C to separate the cytosolic fraction (supernatant) from the pellet containing the membrane fraction. Pellets were resuspended in modified RIPA buffer (50mM Tris-HCl pH 7.4, 1% Triton-X 100, 0.1 % SDS, 0.5 % sodium deoxycholate, 150 mM sodium chloride, 1 mM EDTA pH 8, 1 mM EGTA) plus the inhibitors described above. Protein concentration was quantified as described in [12]. The purity of the fractions was analyzed by western blotting for ERK1 in the cytosolic fraction and transferrin receptor in the membrane fraction. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and western blotted for the proteins of interest. All quantifications for immunoblotted protein levels were carried out with the LI-COR Biosciences Odyssey Infrared Imaging software version 2.

3. Results

3.1. Abi-1 interacts with Cbl upon EGF-stimulation

Cbl proteins are translocated to the plasma membrane upon EGF stimulation [32], and have a role in downregulation of the EGFR and other receptor tyrosine kinases (RTKs), as well as the T-cell receptor (TCR) [9]. In addition to its E3-ligase activity, Cbl can function as an adaptor protein to cluster other important regulators of the endocytic process [7,9]. Cbl was identified as a binding partner for Abi-1 by coimmunoprecipitation using specific Abi-1 antibodies in EGF-stimulated COS7 cells (Fig. 1). The Abi-1/Cbl interaction was readily detected 5 min after EGF stimulation. This interaction was maximal at 15 min, and decreased after 60 min of stimulation (Fig. 1, top panel). The complex between Cbl and Abi-1 was not observed in serum-starved cells (Figure 1, lanes 1 and 2) or in cells growing in serum-containing media (Fig. 1, lanes 9 and 10). The formation of the Abi-1/Cbl complex correlated with the EGF-inducible tyrosine phosphorylation of Cbl (Fig. 1, middle panel). The Abi-1/Cbl complex was also present in the NMuMg epithelial cells after EGF stimulation (data not shown). Thus, EGF induced the formation of a protein complex between Cbl and Abi-1, which may have a role in the regulation of EGFR internalization.

3.2. The Abi-1/Cbl interaction requires the Abi-1 SH3 domain and C-terminal Cbl sequences

We utilized a panel of Cbl mutants in order to identify the Cbl sequences important for binding to Abi-1. The E-3 ligase function of the Ring Finger of Cbl has been shown to be important for EGFR downregulation [33], but additional Cbl sequences are also involved in EGFR internalization [34], as well as EGFR transport from late endosomes to multivesicular bodies and lysosomes [35–37]. We co-expressed EYFP-Abi-1 wild type (WT) with HA-tagged Cbl constructs, including Cbl-WT and various Cbl fragments, such as the Ring Finger domain (RF), the Linker Region (LR), as well as the Cbl N-terminal region which contains the evolutionary conserved Tyrosine Kinase Binding domain (TKB) and the Cbl C-terminal region which contains a Proline Rich domain (PR) that is a likely candidate to interact with the Abi-1 SH3 domain. The Cbl mutants utilized for the experiments are depicted in Fig. 2B. We also utilized two additional Cbl constructs depicted in Fig. 2C: the 70Z Cbl oncoprotein, originally identified from the 70Z/3 pre-B-lymphoma cell line, which fails to ubiquitinate the EGFR upon ligand binding [34], and Cbl C381A, which is also impaired for EGFR ubiquitination [34].

We observed that EYFPAbi-1 wild type (WT) coimmunoprecipitated with wild type HA-tagged Cbl (Fig. 2D, lane 2). Interestingly, we observed greater binding of EYFP-Abi-1-WT to a Cbl mutant (Cbl CR) (Fig. 2B) that encompasses the Ring Finger domain and the Proline Rich Domain (Fig. 2D, lane 6). The increased Abi-1 interaction with this truncated form of Cbl could reflect the existence of inhibitory regions in the Cbl N-terminal sequence, which may partially block binding to Abi-1. We observed weak binding of Abi-1 to a Cbl mutant

containing only the Proline Rich Domain and the C-terminal Leucine Zipper/Ubiquitin Binding Motif (Fig. 2D, lane 5). Thus, the Abi-1/Cbl interaction may be mediated in part by the Cbl PRD and the Abi-1 SH3 domain, but additional sequences appear to be involved in enhancing this interaction. We also detected weak binding of a Cbl-fragment containing the TKB and the Ring Finger (Cbl NR fragment) (Fig. 2D, lane 3). The interaction of Abi-1 with the Cbl fragment containing the Proline Rich Region was much weaker than the interaction between Abi-1 and the Cbl CR C-terminal fragment which included the LR and RF regions in addition to the proline rich domain. Thus, multiple C-terminal Cbl sequences appear to be required for efficient Abi-1 binding. Interestingly, the 70Z Cbl (Fig. 2D, lane 7) and CblC381A (not shown) mutants failed to interact with Abi-1.

Since binding of Cbl to Abi-1 was mediated in part by the Cbl Proline-Rich Domain, we analyzed the role of the Abi-1 SH3 domain in Cbl-binding. Following co-transfection of HA-Cbl with vector control, EYFP-Abi-1 wild type or the EGFP-Abi-1 Δ SH3 mutant, we observed that the Abi-1 SH3 domain is required for the Cbl/Abi-1 interaction as only Abi-1 wild type, but not Abi-1 Δ SH3 coimmunoprecipitated with Cbl (Fig. 2E). Thus, the interaction between Abi-1 and Cbl is mediated in part through an SH3/Proline-Rich domain interaction.

3.3. Abi1 is ubiquitinated by Cbl

Because Abi-1 interacted with the Cbl ubiquitin ligase, we examined whether Abi-1 could be ubiquitinated in a Cbl-dependent manner. We analyzed Abi-1 ubiquitination in the presence of Cbl wild type or various Cbl mutants. We observed that Abi-1 wild type was ubiquitinated in the presence of Cbl wild type but not in the vector transfected cells (Fig. 3, lane 2). Abi-1 ubiquitination was partially retained upon co-expression with a Cbl mutant, Cbl-CR, which contains the Linker Region, Ring Finger Domain and the C-terminal Proline Rich domain (Cbl-CR) (Fig. 3, lane 6). In contrast, Abi-1 was not ubiquitinated upon expression of the E-3 ligase-defective 70Z-Cbl (Fig. 3, lane 7). Interestingly, whereas the closely related Abi-2 protein has been shown to be downregulated by expression of oncogenic tyrosine kinases via the ubiquitin proteasome pathway [38], Abi-1 protein levels do not change upon ubiquitination in cells overexpressing Cbl- wild type (Fig. 3).

3.4. Abi-1 Δ SH3 inhibits EGFR endocytosis

Cbl regulates EGFR endocytosis [10,32,39], and a recent report has implicated Abi-1 in modulation of EGFR endocytosis [13]. Given the EGF-inducible interaction between Cbl and Abi-1, we therefore sought to analyze whether mutation of the Abi-1 SH3 domain affected EGFR internalization. We overexpressed either EYFP-Abi-1 wild type or Abi-1 Δ SH3 in COS7 cells, and analyzed endogenous EGFR endocytosis by visualization of Rhodamine-labeled EGF internalization using confocal microscopy. We consistently observed inhibition of EGFR endocytosis upon overexpression of Abi-1 Δ SH3 but not Abi-1 wild type (Fig. 4).

Quantification of three independent experiments clearly shows that the inhibitory effect of the Abi-1 Δ SH3 is statistically significant. Truncation of N-terminal regions in Abi-1 described previously [19] showed no effect on EGFR internalization (data not shown).

3.5. Abi-1 Δ SH3 inhibits Cbl localization to the plasma membrane

Cbl proteins translocate to the plasma membrane upon EGF-stimulation and accompany the EGFR throughout its endocytic route [32]. We showed that an Abi-1 mutant lacking the SH3 domain required for interaction with Cbl also inhibited EGFR internalization. Thus, we analyzed whether Cbl localization to the plasma membrane upon addition of EGF was altered in the presence of the Abi-1 Δ SH3 mutant. COS7 cells were transfected with vector control, Abi-1 wild type or Abi-1 Δ SH3, and the Cbl accumulation to the plasma membrane was analyzed by microscopy after addition of EGF (Fig. 5A). We observed that expression of Abi-1 Δ SH3 markedly inhibited the EGF-induced translocation of Cbl to the plasma membrane

(Fig. 5A). In contrast, Abi-1 wild type expression did not affect Cbl localization to the membrane after EGF stimulation. Quantification of the number of cells with Cbl at the membrane showed a significant decrease in the percentage of cells that exhibited Cbl accumulation at the membrane in cells expressing Abi-1 Δ SH3, compared to that observed in cells transfected with vector control or Abi-1 wild type (Fig. 5B). Additionally, we employed subcellular fractionation to examine whether Abi-1 Δ SH3 affected the EGF-induced relocalization of Cbl to the plasma membrane. Analysis of three independent experiments showed that upon expression of Abi-1 Δ SH3 there was a decrease in the amount of Cbl localized to the plasma membrane (Fig. 5C and D). Thus, Abi-1 appears to regulate the accumulation of Cbl to the plasma membrane, where it can functionally participate in EGFR endocytosis.

3.6. Kinase active Abl disrupts the Abi-1/Cbl interaction

We previously reported that kinase-active Abl inhibited EGFR endocytosis and one possible mechanism could be through disruption of the Cbl/EGFR interaction [12]. Thus, we tested whether kinase-active Abl could affect the Cbl/Abi-1 complex. We examined complex formation by analysis of Abi-1/Cbl complexes before and after addition of EGF, in the presence of either kinase-active Abl-PP or kinase-inactive Abl-KM. We observed that expression of kinase-active Abl-PP disrupted the EGF-inducible Abi-1/Cbl interaction (Fig. 6, lane 4). In contrast, the ligand-inducible Cbl/Abi-1 complex was stable upon overexpression of kinase-inactive Abl-KM, or in vector transfected cells (Fig. 6, lanes 2 and 6). The disruption of the Cbl/Abi-1 complex by activated Abl-PP may be due to direct tyrosine phosphorylation of Abi-1 and/or Cbl, as both molecules are phosphorylated by activated Abl kinases [15,17,40,41].

4. Discussion

Here we describe a novel complex between Cbl and Abi-1 induced upon EGF-stimulation. The formation of the Abi-1/Cbl complex is coincident with EGF-inducible Cbl tyrosine phosphorylation and its recruitment to the EGFR upon addition of EGF [32]. Downregulation of Abi-1 through RNA interference was reported to inhibit EGFR internalization [13], and this effect was attributed to the loss of an interaction between Abi-1 and N-WASP, a protein linked to EGFR internalization [42]. However, EGFR endocytosis has been shown to be regulated by a multiplicity of mechanisms [2]. Our data suggest that in addition to the Abi-1/N-WASP complex [13], Abi-1 regulates EGFR internalization through its EGF-inducible interaction with Cbl.

Abi-1 has been reported to regulate actin polymerization by formation of complexes with WAVE or N-WASP [13,43]. An intact actin cytoskeleton has been shown to be important for EGFR endocytosis [42,44–46]. Here we propose that Abi-1 modulates EGFR endocytosis through dual pathways: 1) controlling actin polymerization processes required for EGFR endocytosis, and 2) by functioning as an adaptor protein for the recruitment of endocytic regulators such as the Cbl E-3 ligase upon addition of EGF. In contrast to the reported constitutive binding of Abi-1 to N-WASP [13], we find that the interaction between Abi-1 and Cbl is regulated by the addition of EGF. Therefore, the N-WASP/Abi-1 complex may regulate constitutive EGFR endocytosis, whereas the Abi-1/Cbl complex may function inducibly to regulate ligand-stimulated receptor endocytosis.

We found that the Abi-1/Cbl interaction was mediated in part by the Abi-1 SH3 domain and the Cbl Proline Rich Region. Interestingly, two Cbl E-3 ligase defective mutants, 70ZCbl and Cbl 381A, failed to interact with Abi-1, despite the presence of the Cbl Proline-Rich sequences. Thus, sequences other than the proline-rich domain of Cbl appear to be involved in the interaction between Cbl and Abi-1. Additionally, we found that Abi-1 was ubiquitinated in the presence of Cbl. It is possible that ubiquitination of Abi-1 may be required for its interaction with Cbl or that some other Cbl-ubiquitinated proteins function with Abi-1 in the regulation

of EGFR endocytosis. A functional role for ubiquitinated Abi-1 is supported by our observation that Abi-1 is not degraded upon ubiquitination in the presence of Cbl.

EGFR internalization has been shown to be inhibited in some metastatic cancer cells [47]. We have recently reported that oncogenic Abl kinase inhibits EGFR internalization [12]. Here, we found that the complex between Abi-1 and Cbl is disrupted upon expression of constitutive active Abl-PP. Thus, Abl-mediated phosphorylation of Cbl and/or Abi-1 may represent an additional mechanism for the inhibition of EGFR internalization by oncogenic Abl. Activated Abl has been reported to phosphorylate Cbl at tyrosines 674, 731 and 774 [41,48] and to phosphorylate Abi-1 on tyrosine 213 [41]. Phosphorylation of either Cbl or Abi-1 by Abl at specific sites may be required to disrupt the Abi-1/Cbl complex. In this regard, it was reported that the Abl tyrosine kinase disrupts a complex between c-Jun and the Itch ubiquitin ligase, which prevented c-Jun ubiquitination [49]. Thus, Abl can negatively regulate the interaction between specific Abl substrates and their E3-ligases. Abl kinases have been shown to be activated in a subset of metastatic breast cancer cell lines downstream of the EGFR [50]. Consequently, future experiments will examine whether oncogenic Abl kinases regulate the Abi-1/Cbl complex, and thereby modulate endocytosis of the EGFR and other receptors present in metastatic breast cancer cells.

5. Conclusions

In this work, we identify an EGF-inducible complex between the Abi-1 adaptor protein and the Cbl ubiquitin E3 ligase. We show that Abi-1 plays a role in targeting Cbl to the plasma membrane after EGF treatment, and that a mutant Abi-1 protein defective in Cbl binding impairs ligand-induced endocytosis of the EGFR. Moreover, we report that the growth factor-inducible Abi-1/Cbl complex is disrupted by the oncogenic Abl tyrosine kinase, a protein that inhibits EGFR internalization. These findings identify a new role for the Abi-1/Cbl complex in the regulation of ligand-dependent EGFR endocytosis, and highlight the contribution of activated Abl kinases and their downstream targets in the regulation of this process.

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Abbreviations

Abi-1	Abl interacting protein-1
Abl	Abelson tyrosine kinase
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
PBS	phosphate-buffered saline
GFP	green fluorescent protein

HA	hemagglutinin
SDS	sodium dodecyl sulphate
PAGE	polyacrylamide gel electrophoresis
Cbl	Casitas B-lineage
RTK	receptor tyrosine kinase

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Fig. 1. Endogenous Abi-1 forms a complex with Endogenous Cbl upon EGF-stimulation in COS-7 cells

COS7 cells (70 % confluency) were placed in 0.1% FBS for 24 hours and subsequently treated with EGF for the times indicated. Abi-1 was immunoprecipitated with a combination of two polyclonal antibodies (6987/6988) (odd lanes). Normal rabbit serum (NRS) was employed as a negative control (even lanes). Twelve hours prior to serum-starvation, COS7 cells were plated at 2.5×10^6 cells/dish. Lanes 1,2: serum-starved cells. Lanes 3, 4: EGF 5 min. Lanes 5,6: EGF 15 min. Lanes 7,8: EGF 60 min. Lanes 9,10: cells in DMEM 10% FBS. Whole cell lysates showing the levels of Cbl and Abi-1 are shown in the lower panels. These data are representative of 5 independent experiments.

Fig. 2. Analysis of the Cbl/Abi-1 interaction
(A) Diagram of Abi-1 and domains important for protein-protein interactions. (B) Structural diagram of the Cbl mutants utilized. Depicted are the Tyrosine Kinase Binding Domain (TKB) located in the N-terminus and comprised by the 4 helical region (4H), the EF hand and the SH2 domain. (C) Amino acids in the Linker Region and Ring Finger. The residues missing in the 70Z-Cbl mutant are boxed in red and Cysteine 381 is indicated by a purple arrowhead. The Ring Finger Region is underlined in red. (D) **The C-terminal region of Cbl strongly binds Abi-1.** Full-length EYFP-Abi-1 was co-expressed in 293T cells with the indicated HA-tagged Cbl fragments. Lysates were immunoprecipitated with anti-GFP antibody and western blotted with anti-HA antibody to detect coprecipitating Cbl-fragments (upper panel). Levels of EYFP-

Abi-1 and Cbl levels are shown in the middle and lower panels respectively. We observed higher coprecipitation of the HA-Cbl CR mutant in all experiments. The data are representative of 6 independent experiments. * indicates weak binding of Abi-1 with the Cbl-fragments NR and CO. **(D) The Abi-1 SH3 domain is involved in Cbl recruitment.** Wild type HA-Cbl was co-expressed with vector control (lanes 1 and 2), full length EYFP-Abi-1-WT (lanes 3 and 4) or EGFP Abi- Δ SH3 (lanes 5–6) in 293T cells. Lysates were immunoprecipitated with a GFP polyclonal antibody (+) and co-precipitated Cbl was detected by western blotting (upper panel). The same Nitrocellulose membrane was reprobed for GFP to detect Abi-1 levels (lower panel). Anti-Myc polyclonal antibody was employed as a negative control (–). Protein expression in the whole cell lysates is shown in the right panels. These data are representative of 3 independent experiments.

Fig. 3. Abi-1 is ubiquitinated in the presence of Cbl

Wild type EYFP-Abi-1 was coexpressed in 293T cells in the presence of HA-Cbl-WT and the Cbl mutants indicated. Lysates were immunoprecipitated with anti-GFP antibody and blotted for ubiquitin (upper panel). Membranes were reprobed for GFP to detect total Abi-1 levels (middle panel) and for HA to detect the Cbl proteins (lower panel). We observed enhanced ubiquitin signal upon co-expression Abi-1 WT with Cbl-WT (lane 2) and reduced ubiquitination upon co-expression with the Cbl-CR mutant (lane 6). The E-3 ligase-deficient 70ZCbl mutant did not enhance ubiquitination of Abi-1 in the immunoprecipitates (lane 7). These data are representative of three independent experiments.

Fig. 4. Abi1 Δ SH3 inhibits EGFR endocytosis

(A) COS 7 cells were plated onto glass coverslips and 12 hours later were transfected with vector control (EGFP-C1), EYFP Abi-1 WT, or EGFP-Abi-1 Δ SH3. Cells were serum-starved 24 hours after transfection by placing cells in in 0.1 % FBS for 24 hours, and followed by addition of 40 ng/ml EGF- Rhodamine for 15 min at 37 °C. The left panels show GFP protein expression, the middle panels show internalized EGF-Rhodamine, and the right panels show the overlay. Quantification of cells with internalized EGF for three independent experiments is shown in (B).

Fig. 5. Abi-1 ΔSH3 inhibits Cbl localization to the plasma membrane

(A) COS7 cells were transfected with vector control (EGFP), EYFP Abi-1 WT, or EGFP Abi-1 ΔSH3 as indicated. Twenty four hours after transfection, cells were serum-starved for 1 day (left panels) or stimulated with 50 ng/ml of EGF for 2 min (right panel). Transfected cells are shown in green and immunostaining for endogenous Cbl is shown in red. The data are representative of 3 independent experiments. (B) Quantification of the number of cells showing Cbl localized to the plasma membrane before and after EGF stimulation for three independent experiments. After addition of EGF, cells expressing Abi-1 ΔSH3 show significantly lower accumulation of Cbl to the plasma membrane and higher number of cells where Cbl remained cytoplasmically localized. (C) Analysis of Cbl localization to the plasma membrane by subcellular fractionation. COS7 cells were transfected with the indicated constructs (30 and 40% transfection efficiency). Cbl protein levels are shown in the upper panel for vector control and Abi-1ΔSH3-expressing cells; α-tubulin control is shown in the middle panels, and the

levels of Abi-1 Δ SH3 protein are shown in the lower panels. **(D)** Analysis of the fold-change in Cbl protein levels at the plasma membrane for cells expressing vector control or Abi-1 Δ SH3 before and after EGF stimulation for three independent experiments.

Fig. 6. Kinase-active Abl disrupts the Cbl/Abi-1 complex

COS 7 cells were transfected with either vector control (pcDNA), activated Abl-PP or kinase-inactive Abl-KM. At 24 hours post-transfection, cells were serum-starved for 1 day in 0.1% FBS and then stimulated with 100 ng/ml of EGF for 15 min at 37 °C. Lysates (2 mg) were incubated with anti-Abi-1 antibodies, and the co-precipitating Cbl protein was detected by western blotting (upper panel). The levels of immunoprecipitated Abi-1 are shown in the second panel. Note the increase in the molecular weight of Abi-1 in the presence of kinase-active Abl-PP, due to tyrosine phosphorylation. The levels of the indicated proteins present in whole cell lysates are shown in the bottom four panels. These data are representative of three independent experiments.