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ScienceDirect
Cellular Signalling 19 (2007) 1602–1609
CELLULAR SIGNALLING
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Abi-1 forms an epidermal growth factor-inducible complex with Cbl: Role in receptor endocytosis

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Received 26 January 2007; received in revised form 8 February 2007; accepted 9 February 2007
Available online 22 February 2007

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 (ΔSH3) fails to interact with Cbl and inhibits EGFR internalization. We show that expression of the Abi-1ΔSH3 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.

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 Waves 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

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, hemaglutinin; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; Cbl, Casitas B-lineage; RTK, receptor tyrosine kinase.

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doi:10.1016/j.cellsig.2007.02.008

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-1ΔSH3 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-1ΔSH3 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-1ΔSH3 and other Abi-1 mutants utilized were previously described [19]. pCMV2 Abi-1 WT and the mutant pCMV2-Abi-1ΔSH3 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

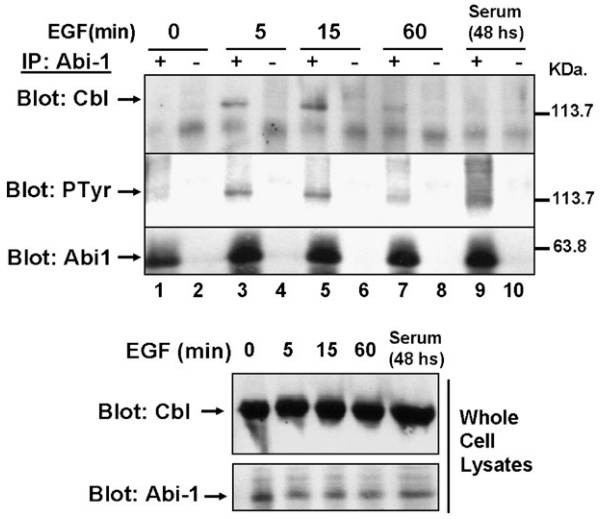

Fig. 1. Endogenous Abi-1 forms a complex with endogenous Cbl upon EGF stimulation in COS-7 cells. COS7 cells (70% confluency) were placed in 0.1% FBS for 24 h and subsequently treated with EGF for the times indicated. Abi-1 was immunoprecipitated with a combination of two polyclonal antibodies (6987/6988) (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.

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 1× 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 with 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 1 day after transfection for 24 h in DMEM containing 0.1% FBS. Cells were placed in ice-cold binding medium (20 mM HEPES–NaOH pH 7.5, 130 mM NaCl, 0.1% bovine serum albumin (BSA)) for 30 min 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,