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The multidomain protooncogenic protein c-Cbl binds to tubulin and stabilizes microtubules

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Abstract

The protooncogenic protein c-Cbl is known to regulate the actin cytoskeleton. In this study, we present results indicating that c-Cbl can also regulate the microtubular network. We have shown that c-Cbl binds to tubulin and microtubules through its tyrosine kinase binding (TKB) domain. However, the character of the interactions described in this report is novel, since the G306E mutation, which disrupts the ability of c-Cbl's TKB to bind to tyrosine-phosphorylated proteins, does not affect the observed interaction between c-Cbl and microtubules. Furthermore, overexpression of c-Cbl in human pulmonary artery endothelial cells and COS-7 cells leads to microtubule stabilization. We demonstrate that this effect of c-Cbl is mediated by TKB, and, like c-Cbl binding to microtubules, is independent of the ability of TKB to bind to tyrosine-phosphorylated proteins. Finally, we have shown that c-Cbl directly polymerizes microtubules in vitro, and that TKB is necessary and sufficient for this effect of c-Cbl. In this last phenomenon, as well as in the previous ones, the effect of TKB is not sensitive to the inactivating G306E mutation. Overall, the results presented in this report suggest a novel function for c-Cbl—microtubule binding and stabilization.

Keywords: c-Cbl; Tubulin; Microtubules; Polymerization; Stabilization; Acetylation; Endothelial cells

Introduction

Microtubules are a major component of the cytoskeleton and play essential roles in many cellular processes including mitosis, intracellular transport, cell morphology, and motility. Microtubules are composed of αβ-tubulin heterodimers, which assemble in a head-to-tail manner to form a linear protofilament. Protofilaments are subsequently joined through lateral interactions to form the wall of the cylindrical microtubule (reviewed in [1]). Microtubules are highly dynamic, constantly switching between phases of growth and shrinkage mediated by the addition or loss of tubulin dimers from the ends of the microtubule; this property of microtubules known as dynamic instability [2,3] is based on binding and hydrolysis of GTP by tubulin subunits (reviewed in [4]). The assembly and stability of microtubules is regulated not only by the nucleotide state of tubulin, but also by interactions with cellular factors like microtubule-associated proteins (MAPs) and other regulatory proteins (reviewed in [5]).

c-Cbl is a ubiquitously expressed cytoplasmic protein, which has several distinct domains including a tyrosine kinase-binding (TKB) domain, a RING finger, a proline-rich region, a cluster of tyrosine phosphorylation sites and a leucine zipper/ubiquitin-associated domain. The N-terminal TKB domain is highly conserved and consists of a four-helix bundle, a calcium-binding EF-hand and a modified

Abbreviations: DSP, Dithiobis[succinimidylpropionate]; GST, glutathione-S-transferase; HPAEC, Human pulmonary artery endothelial cells; MAP, Microtubule-associated proteins; PTK, protein tyrosine kinase; TKB, tyrosine kinase-binding domain.

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SH2 domain (reviewed in [6–8]). TKB binds to phosphotyrosine residues of multiple protein tyrosine kinases (PTKs) [9–13]. c-Cbl has several functional roles, acting both as a multivalent adapter protein and as an inhibitor of various PTKs (reviewed in [6,8]). The latter function is linked to its E3 ubiquitin ligase activity [14–16].

Co-precipitation of c-Cbl with tubulin from B-cell lysates has been shown previously using colchicine-agarose [17] and immunoprecipitation from cells treated with DSP, a membrane-permeable cleavable cross-linking reagent [18]. However, the mode of interactions between c-Cbl and tubulin has not been revealed in these studies. In the course of our recent study that involved identification of c-Cbl-associated proteins from T cells, we have shown that c-Cbl co-purifies with α- and β-tubulin [19]. In this report, we confirm this result by demonstrating that c-Cbl co-immunoprecipitates with α- and β-tubulin and binds to microtubules. This binding is mediated by the N-terminal region of c-Cbl containing its TKB domain, but is not affected by mutational inactivation of this domain. Since c-Cbl interacts with tubulin and microtubules, we were prompted to evaluate the effect of c-Cbl on microtubule stability. Tubulin is known to undergo several post-translational modifications that accumulate on stable microtubules. These include acetylation [20], phosphorylation [21], polyglycylation [22], polyglutamylation [23], and the generation of non-tyrosinatable tubulin [24,25]. Our results demonstrate that overexpression of c-Cbl in COS-7 cells leads to an increase in tubulin acetylation, suggesting that c-Cbl increases microtubule stability. Although the N-terminal region of c-Cbl has been found to be required for this function, the G306E mutation, which prevents binding of TKB to activated PTKs thus disrupting E3-related functions of c-Cbl, does not diminish the microtubule-stabilizing effect of c-Cbl. In addition, we have shown that c-Cbl overexpressing human pulmonary artery endothelial cells (HPAEC) increase their resistance to the nocodazole-induced depolymerization of microtubules. Furthermore, we have shown the ability of c-Cbl to polymerize microtubules in vitro. Consistent with our earlier results, the N-terminal region of c-Cbl alone is capable of polymerizing microtubules in vitro acting in a G306E mutation-independent fashion. Overall, the data presented in this report suggest that c-Cbl may have an additional novel function related to binding to and stabilization of microtubules.

Materials and methods

Cells and transfection

COS-7 cells were grown in DMEM containing 10% fetal bovine serum and other supplements as described previously [26]. COS-7 cells were transiently transfected using LipofectAMINE 2000 (Life Technologies, Inc., Grand Island, NY) following manufacturer's recommendations. JMC-D3, a Jurkat-derived clone overexpressing wild-type c-Cbl was kindly provided by Dr. H. Band (Northwestern University School of Medicine, Evanston, IL) and propagated in RPMI-1640 containing 10% fetal bovine serum and other supplements as described previously [10]. Human pulmonary artery endothelial cells (HPAEC) (Clonetics, BioWittaker Inc. Frederich, MD) were propagated in culture medium EGM-2 (Clonetics, BioWittaker Inc.) and used at passages 6–10. HPAEC were transiently transfected using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) as described previously [27].

Plasmids

For mammalian expression of wild-type c-Cbl, pAlterMAX plasmid encoding HA-tagged wild-type c-Cbl was used. pAlterMAX plasmids encoding wild-type c-Cbl and v/c-Cbl have been described previously [26]. pAlterMAX plasmids encoding v/c-[G306E]Cbl and [358–906]Cbl were kindly provided by Dr. H. Band. The pGEX2T vectors expressing glutathione-S-transferase (GST)-fused wild-type c-Cbl was kindly provided by Dr. Y. Yarden (Weitzmann Institute, Rehovot, Israel). The pGEX2T vectors expressing GST-v/c-Cbl, GST-v/c-[G306E]Cbl and GST-[358–906]Cbl were kindly provided by Dr. H. Band.

Antibodies

The affinity-purified polyclonal antibodies against c-Cbl, α-tubulin and HA, normal rabbit IgG and anti-α-tubulin mouse monoclonal antibody (TU-02) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG were purchased from Molecular Probes (Eugene, OR). Mouse monoclonal antibodies to β-tubulin (TUB2.1) and acetylated tubulin (6-11B-1) were purchased from Sigma (St. Louis, MO). The anti-acetylated tubulin mouse monoclonal antibody (6-11B-1) used with HPAEC was purchased from Accurate Chemicals and Scientific Corporation (Westbury, NY). Mouse monoclonal antibodies to β-catenin (clone 14), Grb2 (clone 81) and Smad 2/3 (clone 18) were purchased from BD Biosciences (San Diego, CA). The polyclonal antibody to myosin A was purchased from Covance (Richmond, CA). Mouse monoclonal antibody to GAPDH was purchased from Research Diagnostic (Flanders, NJ). Horseradish peroxidase-conjugated secondary antibodies against rabbit and mouse IgG were purchased from Amersham Biosciences (Piscataway, NJ). The affinity-purified donkey anti-mouse IgG conjugated to Texas Red was purchased from Jackson ImmunoResearch laboratories (West Grove, PA).

Immunoprecipitation and immunoblotting

Cells were treated with 0.3 mg/ml DSP (Dithiobis[succinimidylpropionate]) purchased from Pierce Biotechnology (Rockford, IL), where indicated, for 1 h at room temperature