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CAP (Cbl associated protein) regulates receptor-mediated endocytosis

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ABSTRACT

CAP (c-Cbl associated protein)/ponsin belongs to a family of adaptor proteins implicated in cell adhesion and signaling. Here we show that CAP binds to and co-localizes with the essential endocytic factor dynamin. We demonstrate that CAP promotes the formation of dynamin-decorated tubule like structures, which are also coated with actin filaments. Accordingly, we found that the expression of CAP leads to the inhibition of dynamin-mediated endocytosis and increases EGFR stability. Thus, we suggest that CAP may coordinate the function of dynamin with the regulation of the actin cytoskeleton during endocytosis.

Structured summary:
MINT-6804322: *CAP* (uniprotkb:Q9BX66) *physically interacts* (MI:0218) with *Cbl* (uniprotkb:Q8K4S7) and *dynamin 2* (uniprotkb:P39052) by *pull down* (MI:0096)
MINT-6804285: *CAP* (uniprotkb:Q9BX66) *physically interacts* (MI:0218) with *FAK* (uniprotkb:O35346), *vinculin* (uniprotkb:P85972) and *dynamin 2* (uniprotkb:P39052) by *pull down* (MI:0096)
MINT-6804245, MINT-6804259, MINT-6804272: *CAP* (uniprotkb:Q9BX66) *physically interacts* (MI:0218) with *dynamin 2* (uniprotkb:P39052) by *pull down* (MI:0096)
MINT-6804344: *CAP* (uniprotkb:Q9BX66) *physically interacts* (MI:0218) with *dynamin 2* (uniprotkb:P50570) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-6804371: *dynamin 1* (uniprotkb:P21575) *physically interacts* (MI:0218) with *CAP* (uniprotkb:O35413) by *anti bait coimmunoprecipitation* (MI:0006)
MINT-6804446, MINT-6804464: *F-actin* (uniprotkb:P60709), *CAP* (uniprotkb:Q9BX66) and *dynamin 2* (uniprotkb:P50570) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)

1. Introduction

Vinexin, CAP (c-Cbl associated protein)/ponsin, and ArgBP2 (Arg Binding Protein 2) belong to a family of adaptor proteins characterized by the presence of an NH2-terminal sorbin homology (SoHo) region and three COOH-terminal SH3 (Src homology domain 3) [1]. The sorbin domain has been recently suggested to operate as a protein–protein interaction motif that binds to lipid raft enriched proteins [2] and to cortical cytoskeleton proteins [3].

CAP/ponsin (hereafter referred to as only CAP) was independently identified as a protein associated to the signaling ubiquitin ligase Cbl [4] and as a component of the adherens junctions connected to the nectin–afadin system [5]. Recently, CAP has been implicated in the insulin signaling [6], although its exact role in the pathway is still controversial [7].

Here we show that CAP binds to the major endocytic factor dynamin and affects the actin cytoskeleton organization. Accordingly, we found that CAP expression impairs receptor-mediated endocytosis.

2. Materials and methods

2.1. Plasmids

The cDNA encoding for mouse FLAG-tagged CAP was a generous gift of Alan R. Saltiel (University of Michigan).

2.2. Antibodies

The antibodies used in this study are: rabbit polyclonal anti-CAP (Upstate-Millipore, Temecula, CA, USA), mouse monoclonal anti-vinculin (Sigma–Aldrich, St. Louis, MO, USA), mouse monoclonal anti-FAK (Upstate-Millipore), mouse monoclonal anti-dynamin1

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(Hudy1, Upstate-Millipore) and DG1 (De Camilli, Yale University, Connecticut, USA), goat anti-dynamin2 (C-18, Santa Cruz, CA, USA), rabbit anti-dynamin2 (McNiven, Mayo Clinic, MN, USA), mouse monoclonal anti-FLAG, mouse monoclonal anti-FLAG conjugated beads and rabbit polyclonal anti-FLAG (Sigma–Aldrich), rabbit anti-Cbl (C-15, Santa Cruz), mouse monoclonal anti-EGFR (Millipore, USA), rabbit anti-EGFR (Di Fiore, IFOM, Milan, Italy), anti-phospho-MAPK (p42/44), anti-phospho-AKT (Ser 473), anti-AKT (Cell Signaling).

2.3. Cell culture and immunofluorescence microscopy

HEK293, HeLa and COS-7 cells were purchased from ATCC. Cells were grown in standard Dulbecco's modified Eagle's medium (GIBCO-Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (GIBCO-Invitrogen).

Primary cultures of osteoclasts were prepared as previously described [8]. Cell transfections were performed with Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Immunofluorescence staining experiments were performed as previously described [9]. Oregon Green and Texas Red-conjugated secondary antibodies and Texas Red-conjugated phalloidin were purchased (Molecular Probes-Invitrogen).

Fluorescence was visualized with Axiophot epifluorescent microscope (Carl Zeiss Inc., Thornwood, NY) using 40× and 63× oil-immersion objectives.

2.4. EGF and transferrin internalization assays

Internalization assay of fluorescently conjugated ligands was performed in HeLa cells as described [10,11]. Receptor internalization block was measured in two independent experiments, counting at least 100 cells per condition in duplicate.

In the biotinylation assay HEK293 cells were starved for 16 h and incubated with reactive NHS-SS-biotin compound (0.5 mg/ml) (Pierce, Rockford, IL, USA) dissolved in tyrode buffer (10 mM HEPES, 136 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 1.3 mM $MgCl_2$) for 3 min at 37 °C. After two washes in tyrode buffer, cells were incubated for 10 min at 37 °C in Dulbecco's modified Eagle's serum-free medium plus EGF (20 ng/ml) (Upstate-Millipore). Cells were kept on ice for 1 hour in reducing buffer (100 mM DTT, 150 mM NaCl; 100 mM TRIS pH 8,8) and incubated in ice cold blocking buffer [50 mM iodoacetamide (Sigma–Aldrich), 250 mM Tris pH 8] for 30 min. Finally, cells were lysated in ice cold RIPA buffer [Tris 20 mM pH 7.5, NaCl 100 mM, NaF 50 mM, NP40 1%, DOC 0.1%, SDS 0.1%, EDTA

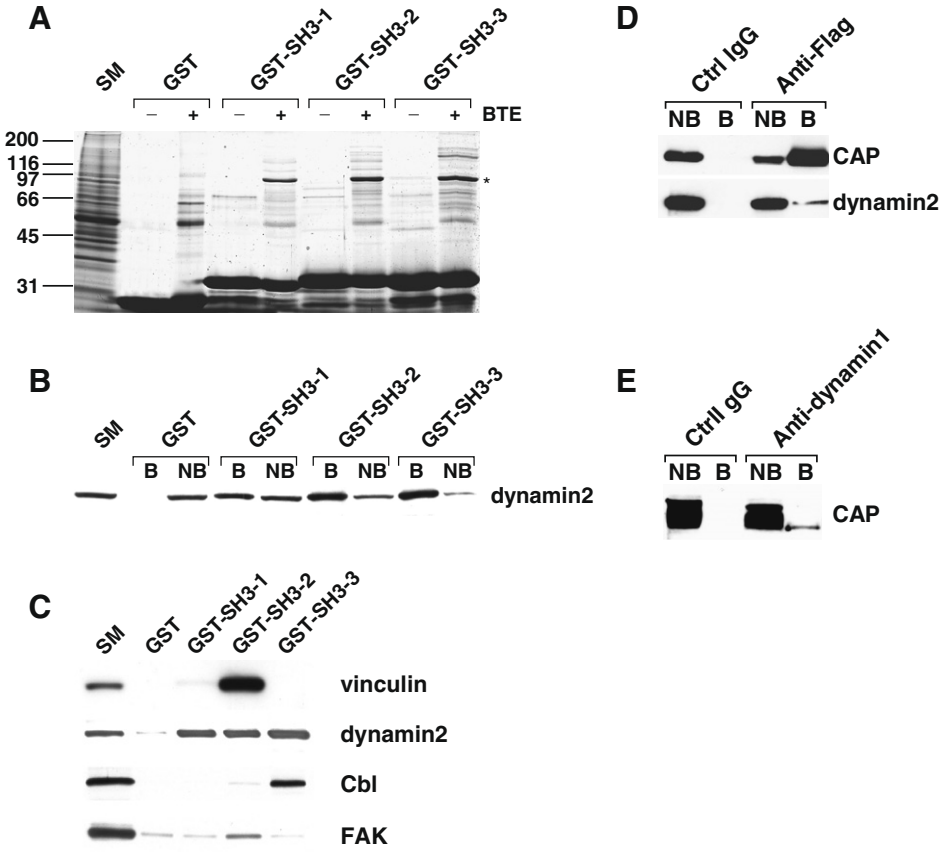

Fig. 1. (A) CAP binds to dynamins. The three SH3 domains of CAP, fused to the GST domain, were used in affinity purification experiments with rat brain extract (BTE, brain Triton X-100 extract). Pulled down proteins and GST fusion SH3 domains incubated with no extract were resolved by SDS–PAGE and stained with Coomassie Blue. Asterisk marks the position of dynamin protein. (B) Anti-dynamin2 rabbit polyclonal antibody was used in western blotting to detect dynamin2 in protein material bound to each CAP GST-SH3 domain (SM, starting material; B bound material; NB, unbound material). (C) The amount of dynamin2 and other CAP interacting proteins, retained by the three GST-SH3 domains and by the GST as control, was compared by western blotting analysis using the indicated antibodies. (D) Immunoprecipitation of FLAG-tagged CAP from HEK293 cells expressing FLAG-CAP and dynamin2-HA. Total mouse immunoglobulins (IgG) were used as a negative control of the immunoprecipitation experiments. Presence of CAP and dynamin2 in the immunoprecipitated proteins retained by the anti-FLAG conjugated beads was detected by western blotting using anti-CAP and anti-dynamin2 rabbit polyclonal antibodies. (E) Immunoprecipitation of endogenously expressed dynamin1 from rat cerebellum extract, utilizing the anti-dynamin1 mouse monoclonal antibody DG1. Mouse total immunoglobulins were used as a negative control of the immunoprecipitation experiments. Immunoprecipitated protein material was assayed by western blotting using anti-CAP rabbit polyclonal antibody.