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Interaction of activated Rab5 with actin-bundling proteins, L- and T-plastin and its relevance to endocytic functions in mammalian cells

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ABSTRACT

Rab5 is a GTP-binding protein that is crucial for endocytic machinery functions. We previously identified L-plastin as a binding protein for Rab5, using an affinity column with constitutively active Rab5. L- and T-plastin are isoforms of a plastin protein family belonging to actin-bundling proteins that are implicated in the regulation of cell morphology, lamellipodium protrusion, bacterial invasion and tumor progression. However, the physiological relevance of Rab5 binding to plastin has remained unclear. Here, we show that L- and T-plastin interacted only with activated Rab5 and that they co-localized with Rab5 on the plasma membrane and endosome. Rab5 activity was also higher in both L- and T-plastin over-expressing Cos-1 cells. Furthermore, expression of L- and T-plastin increased the rate of fluid-phase endocytosis. These findings imply that the Rab5 is either activated or the activity is sustained by interaction with plastin, and that this interaction influences endocytic activity.

1. Introduction

Endocytosis is an essential mechanism for several important physiological processes such as uptake of nutrients and control of extracellular signaling [1,2]. Rab5 is a 25-kDa small GTP binding protein that is involved in clathrin-dependent endocytosis, phagocytosis and macropinocytosis [3]. Rab5 cycling between the GDP- (inactive) and GTP-bound (active) forms is a process tightly controlled by GTPase-activating proteins (GAPs), guanine nucleotide-exchange factors (GEFs) and GDP dissociation inhibitors (GDIs) [4,5]. Thus, it is understood that the activity of Rab5 is highly regulated by the interaction with other proteins.

In our previous study, we identified caveolin-1 as a Rab5 associating protein [6]. Interestingly caveolin-1 increased Rab5 activity when caveolin-1 was over-expressed in Cos-1 cells, consequently enhancing caveolae mediated endocytosis. At the same time, we also identified several Rab5 associating proteins by liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS), carried out after the proteins were isolated by a constitutively activated Rab5 mutant, Rab5Q79L affinity chromatography [7]. Among these proteins, an actin bundling protein, L-plastin, was identified as a novel Rab5 associating protein.

Plastin (also called fimbrin) is a 68-kDa protein that has the ability to bundle microfilaments *in vitro*. It belongs to a class of actin-bundling proteins that has been conserved from lower eukaryotes to humans. Its most extensively characterized isoforms, known as T- and L-plastin, show 80% identity at the amino acid level but display significant differences in function [8]. These two isoforms are differentially expressed and both play roles in actin filament organization in a cell type-specific manner. L-plastin is expressed in hematopoietic cells such as lymphocytes and macrophages, and is also expressed in neoplastic cells of non-hematopoietic origins [9,10].

However, the role of the plastin–Rab5 interaction has not been reported, and it has not been demonstrated whether it has any impact to endocytic pathways. Thus, in order to understand the physiological relevance of the interaction of Rab5 with plastin, we investigated the intracellular localization of plastin and their effect on Rab5 activity.

2. Materials and methods

2.1. Antibodies

Antibodies were obtained from the following sources: monoclonal anti-HA, polyclonal anti-rabbit HA, anti-mouse IgG-cy3, and

Abbreviations: GAPs, GTPase-activating proteins; GEF, guanine nucleotide-exchange factors; GDI, GDP dissociation inhibitors; LC–MS/MS, liquid chromatography–mass spectrometry/mass spectrometry; R5BD, Rab5 binding domain.

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anti-mouse IgG-cy3 from Sigma–Aldrich (St. Louis, MO); anti-L-plastin and anti-T-plastin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-Rab5 and anti-caveolin-1 were obtained from BD Bioscience (San Diego, CA) and anti-GFP was from Novus Biologicals (Littleton, CO).

2.2. Cell culture

Cos-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM: Invitrogen Novagen, Milwaukee, WI) supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin (DMEM–10%FBS–1%PS). HEK293T cells were also cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin and 1% streptomycin (DMEM–10%FBS–1%PS) [6].

2.3. Expression vector constructs

The GFP-pcDNA3 vector was a kind gift from Dr. Y. Mitsuuchi (Temple University School of Pharmacy, Philadelphia, PA, USA). GFP-tagged constitutively activated Rab5 mutant (GFP-Rab5Q79L), constitutively inactivated Rab5 mutant (GFP-Rab5S34N) and wild type Rab5 (GFP-Rab5wt) were obtained as previously described [6]. The full length L- and T-plastin in pT7Blue plasmids were constructed as described previously [11,12]. The L- and T-plastin DNA were then amplified by PCR methods using specific primers and cloned into pcDNA3.1 and GFP-pcDNA3. The GST-R5BD vector was kindly provided by Dr. G. Li (University of Oklahoma Health Science Center, Oklahoma City, OK, USA) [13].

2.4. Immunoprecipitation

First, to investigate whether Rab5 activity influences the binding of L- and T-plastin, cells were co-transfected with the mutant Rab5 (Rab5Q79L or Rab5S34N) expression vector together with either L-plastin or T-plastin expression vector using Lipofectamine 2000 reagent, as described by the manufacturer (Invitrogen). After 48 h, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and were lysed for 30 min at 4 °C with buffer (10 mM Tris, pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml PMSF, 7.6) containing either 5 μM GTPγS (for Rab5Q79L) or 5 μM GDP (for Rab5S34N). The clarified lysates were incubated with anti-GFP antibodies for 1 h at 4 °C. The complexes were precipitated with protein A-Sepharose (Sigma–Aldrich) for 1 h at 4 °C and were washed extensively with lysis buffer containing either 5 μM GTPγS (for Rab5Q79L) or 5 μM GDP (fro Rab5S34N). The beads were resuspended in SDS sample buffer, and subjected to SDS–PAGE with running buffer (25 mM Tris–HCl, 250 mM glycine, 0.1% SDS), followed by Western analysis using T-plastin or L-plastin specific antibodies.

2.5. Immunostaining

Cos-1 cells were co-transfected with GFP-tagged wild type Rab5 with L-plastin or T-plastin. After 48 h, the cells were fixed with 4% formaldehyde in PBS for 10 min. After nonspecific binding of antibodies was blocked by 10% sheep serum for 60 min, cells were incubated with primary antibody in 10% sheep serum for 60 min. Bound primary antibodies were visualized with a secondary antibody. Slide glasses were thereafter mounted with IMMU-Mount (Thermo Scientific, Pittsburgh, PA). Cells were observed with confocal fluorescence microscopy (OLYMPUS, FV500-IX).

2.6. Analysis of Rab5 activity

The GST-R5BD (Rab5 binding domain) pull down method based on a report by Liu et al. [13] was performed. GST-R5BD was purified from *Escherichia coli* (BL-21 codon plus (DE3) RIL) according to standard procedures. Cells were washed twice with PBS and lysed for 5 min in 1 ml of lysis buffer (25 mM Hepes, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% NP-40, 2% glycerol, 1 mM DTT, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml PMSF). Lysis extract was clarified by centrifugation and lysed for 5 min in 1 ml

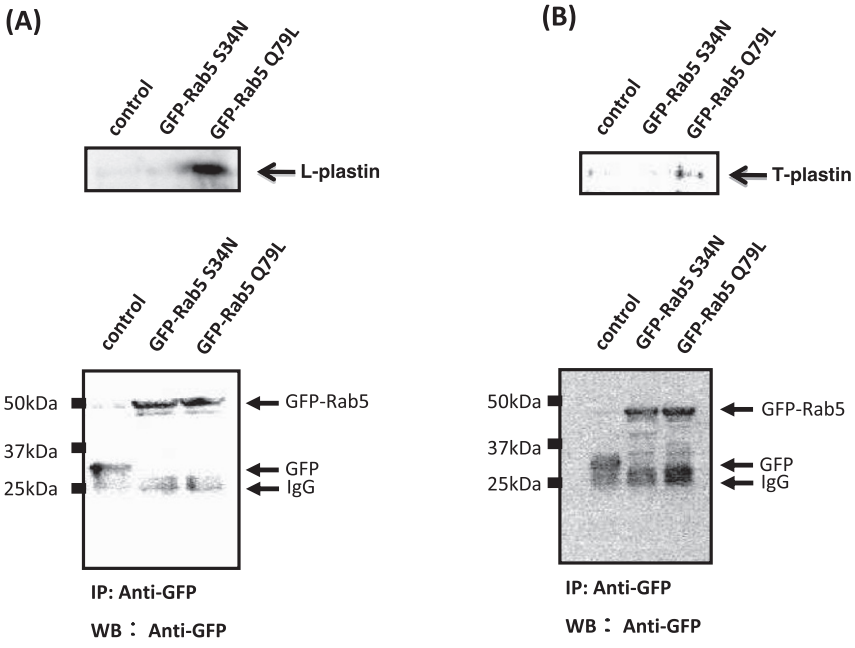

Fig. 1. T- and L-plastin interacted with activated Rab5. Cells were transfected with the mutant Rab5 expression vector (GFP-Rab5Q79L or GFP-Rab5S34N) together with either L-plastin or T-plastin. After preparing the cell extract lysate, Rab5 was immuno-precipitated with anti-GFP antibodies and subjected to SDS–PAGE, followed by Western analysis using T-plastin (A) or L-plastin (B)-specific antibodies. The lower panels show that an equal amount of Rab5 was immunoprecipitated.