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Mobility of tethering factor EEA1 on endosomes is decreased upon stimulation of EGF receptor endocytosis in HeLa cells

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ABSTRACT

Tethering factor EEA1, mediating homotypic fusion of early endosomes, was shown to be localized in membrane-bound state both in serum-deprived and stimulated for EGF receptor endocytosis cells. However, it is not known whether dynamics behavior of EEA1 is affected by EGF stimulation. We investigated EEA1 cytosol-to-membrane exchange rate in interphase HeLa cells by FRAP analysis. The data obtained fitted two-states binding model, with the bulk of membrane-associated EEA1 protein represented by the mobile fraction both in serum-starved and EGF-stimulated cells. Fast recovery state had similar half-times in the two cases: about 1.6 s and 2.8 s, respectively. However, the recovery half-time of slowly cycled EEA1 fraction significantly increased in EGF-stimulated comparing to serum-starved cells (from 21 to 99 s). We suppose that the retardation of EEA1 fluorescence recovery upon EGF-stimulation may be due to the increase of activated Rab5 on endosomal membranes, the growth of the number of tethering events between EEA1-positive vesicles and their clustering.

1. Introduction

Homotypic early endosome fusion is one of the main processes during endosome maturation. Multiple fusions result in increase of endosome surface, thus creating an “excess” of endosomal membrane. This extra membrane provides the possibility to form invaginations and further fission of numerous vesicles into the endosome lumen. Endosomal membrane domains undergoing this process are able to concentrate membrane-bound cargo, such as EGF receptors, thus facilitating the cargo transition inside an endosome and its selective degradation after endosome–lysosome interaction.

Early endosomal antigen 1 (EEA1) protein is known to mediate tethering of early endosomes, the process which precedes their fusion [1]. EEA1 molecules form a parallel coil-coiled homodimer, composed of two 170 kDa subunits [2]. C-terminal region of each EEA1 monomer has RBD domain that interacts with GTP-bound form of small GTPase Rab5 [3] and FYVE domain, which recognizes phosphatidylinositol 3-monophosphate (PI3P) [4,5]. EEA1 has also zinc finger domain on its N-terminus that can bind Rab5 [3]. It was proposed that EEA1 associates with endosomal membrane by C-terminal region, while its N-terminus is oriented perpendicularly to the membrane. This configuration allows EEA1 to act as the tethering factor, by binding Rab 5, localized at the adjacent early endosome [6].

PI3P was shown to be critical for EEA1 binding and tethering [7,8], but Rab5 is necessary for fusion [8]. However, the role of Rab5 is dual as in active, GTP-bound form, Rab5 was shown to recruit PI3-kinase Vps34, which generates PI3P-enriched regions on endosomal membrane [9]. In its turn, Rab5 activation can be triggered by exchange factor RIN1 that is stimulated by EGF-receptor (EGFR) tyrosine kinase upon the formation of EGF-EGFR complexes and their internalization [10,11].

Altogether, abovementioned data resulted in current concept, according to which EEA1 should be recruited to endosomal membrane from cytoplasm upon EGF-receptor endocytosis stimulation. However, immunofluorescent analysis shows mostly vesicular

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localization of EEA1 protein even for cells preincubated in serum-deprived medium for 12–24 h to deplete serum growth factors and minimize receptor-mediated ligand-induced endocytosis [12–14], in regulation of which EEA1 seems to be specifically involved [15]. Our recent findings indicate that these EEA1 vesicles are unlikely the endosomes formed during constitutive endocytosis [14] or autophagosomes [16].

Thus, the vesicular localization of EEA1 protein may be not linked ultimatively to the presence of cargo within the EEA1 vesicle. However, it could be suggested that characteristics of EEA1 membrane association may be different before and after stimulation of specific cargo endocytosis. Such possibility is supported by the finding that EEA1 has higher recovery half-time and increased immobile fraction in interphase comparing to mitotic MDCK cells [17]. However, it is not known whether endocytosis stimulation of such cargo as EGF receptor affect binding dynamics of EEA1 at the EEA1-positive vesicles in interphase cells. To answer this question, we employed fluorescence recovery after photobleaching (FRAP) analysis to estimate the mobile fraction and compare the rate of EEA1 cytosol-to-membrane cycling under serum deprivation for 12 h and after stimulation of EGF receptor endocytosis in HeLa cells.

2. Materials and methods

2.1. Cell culture, plasmid construct and transfection

HeLa cells were obtained from the Russian Cell Culture Collection (Institute of Cytology, St. Petersburg, Russia). The cells were grown in atmosphere of 5% CO2 in DMEM supplemented with 10% fetal bovine serum at 37 °C.

For experiments the cells were transiently transfected with the plasmid encoding N-terminal EGFP-tagged EEA1 protein, which was a gift from Dr. Silvia Corvera (Addgene plasmid # 42307) [18]. Briefly, the cells were split on 4-well μ-slides (Ibidi, Denmark). Then $1{,}5 \times 10^5$ cells were thansfected with 0.5 μg of plasmid DNA using lipofectamine 3000 (Invitrogen, USA), according to manufacturer's instructions. Transfected cells were grown in DMEM, supplemented with 10% fetal bovine serum for 24 h, after that cells were serum starved by shifting to DMEM media, containing 0.1% serum for 12 h before experiment.

2.2. Stimulation of EGFR endocytosis

The EGF receptor endocytosis was stimulated according to pulse-chase protocol. Briefly, the cells were washed twice with warm (37 °C) working medium (WM) consisting of DMEM supplemented with 20 mM HEPES and 1% BSA. The endocytosis was stimulated by addition of 2 nM biotinylated EGF (Invitrogen, USA) in fresh WM at 37 C for 5 min. For FRAP experiments biotinylated EGF were preliminarily conjugated with streptavidine-Cy3 (Invitrogen, USA) to achieve 1EGF:1streptavidin tetramer ratio as described earlier [19].

2.3. Immunofluorescent microscopy

The cells cultured on coverslips were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 15 min and incubated in 1% BSA for 30 min. After that the cells were incubated with monoclonal mouse antibody specific to EEA1 (Cat. № 610457, Transduction Lab, USA) at dilution 1:200 in 1% BSA for 1 h at room temperature and then with secondary antibody GAM-Cy3 (Jackson ImmunoResearch, USA) in dilution 1:200 for 20 min at 37 °C. Finally, the cells were mounted in 0.2 M DABCO (1.4-diazabicyclo(2.2.2)octane) (Sigma–Aldrich, USA) glycerol-containing media.

Images were obtained using Leica TCS SP5 confocal microscope (Leica Microsystems, Germany), equipped with solid-state lasers for excitation (488, 543 nm) and HCX PL APO CS 40X oil immersion objective (NA = 1.3, Leica). Images were taken by sequential scanning mode. EGFP fluorescence was exited at 488 nm and registered in the 495–530 nm range (green channel). Cy3 fluorescence was exited at 543 nm and registered in the 570–650 nm range (red channel).

2.4. FRAP analysis

FRAP experiments were performed in FluoroBrite DMEM media (Life Technologies) at 37 °C. The focal plane was chosen in the basal part of the cells, where majority of EEA1-vesicles is localized. Prebleach images (512 × 512 pixels) were acquired using 488- and 543 nm laser lines at low laser power. The scanning rate was 400 Hz, pinhole diameter - 4 Airy units. Circular region of 4 μm in diameter (about 2-fold exceeding the size of vesicles), was photobleached by scanning during 5 frames with 488 nm argon laser at full power. Then fluorescence recovery was monitored with prebleach settings during 150 frames with 0.7 s intervals.

Postbleach fluorescence intensity of vesicle was determined by manual tracking using ImageJ 1.40 g (National Institute of Health, USA) software. Vesicles, which stayed in focal plane and did not undergo fusion or fission during 50–150 frames (35–105 s), were included in further analysis. Obtained data were background subtracted, corrected for unintentional photobleaching and then normalized to prebleach intensity and bleaching depth, as described in Ref. [20]. Curves were fitted with one or two exponential term equations and fluorescence recovery half-time (t_h) and mobile fraction were calculated [21,22].

2.5. Statistics

All experiments were repeated at least 3 times. Statistical analysis was performed using R software [23]. Significant differences were determined with the Mann–Whitney U test. Data are presented as means ± SEM, if other is not indicated.

3. Results and discussion

3.1. Characterization of EEA1-vesicles taken into FRAP analysis

To perform FRAP analysis, HeLa cells were transiently transfected with plasmid, encoding N-terminal EGFP-tagged EEA1 protein (EGFP-EEA1). We validated the construct by comparing the cellular distribution of EGFP-EEA1 and endogenous EEA1 protein, stained with antibodies at fixed cells (Fig. 1A). As can be seen, the intracellular distribution of the two labels is similar. Earlier we have shown that EEA1-vesicles are different in the level of bound EEA1 and can be divided into two fractions: strongly decorated with EEA1 (bright) and slightly stained by EEA1 antibody (dim) [14]. Practically all bright EEA1 vesicles have dual labeling, but only about half of dim EEA1-vesicles, visualized by antibody staining, are revealed also by EGFP-EEA1 construct (Fig. 1A). We suggest that the expression level of the plasmid results in only partial replacement of endogenous protein on the membrane, so that EGFP moiety added to N-terminus of EEA1 does not prevent normal tethering that is rescued by endogenous EEA1. However, this proportion is insufficient to reliably visualize EGFP-EEA1 on dim vesicles and, consequently, to monitor their fluorescence recovery after photobleaching. Thus, for FRAP experiment we considered only bright EEA1 vesicles.

Next, to characterize the behavior of EEA1-vesicles in live cells, we tracked randomly selected bright EEA1-vesicles for 2 min