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Interaction with epsin 1 regulates the constitutive clathrin-dependent internalization of ErbB3

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ABSTRACT

Background: In contrast to other members of the EGF receptor family, ErbB3 is constitutively internalized in a clathrin-dependent manner. Previous studies have shown that ErbB3 does not interact with the coated pit localized adaptor complex 2 (AP-2), and that ErbB3 lacks two AP-2 interacting internalization signals identified in the EGF receptor. Several other clathrin-associated sorting proteins which may recruit cargo into coated pits have, however, been identified, and the study was performed to identify adaptors needed for constitutive internalization of ErbB3.
Methods: A high-throughput siRNA screen was used to identify adaptor proteins needed for internalization of ErbB3. Upon knock-down of candidate proteins internalization of ErbB3 was identified using an antibody-based internalization assay combined with automatic fluorescence microscopy.
Results: Among 29 candidates only knock-down of epsin 1 turned out to inhibit ErbB3. Epsin 1 has ubiquitin interacting motifs (UIMs) and we show that ErbB3 interacts with an epsin 1 deletion mutant containing these UIMs. In support of an ErbB3-epsin 1 UIM dependent interaction, we show that ErbB3 is constitutively ubiquitinated, but that both ubiquitination and the ErbB3-epsin 1 interaction increase upon ligand binding.
Conclusion: Altogether the results are consistent with a model whereby both constitutive and ligand-induced internalization of ErbB3 are regulated through interaction with epsin 1.
General significance: Internalization is an important regulator of growth factor receptor mediated signaling and the current study identify mechanisms regulating plasma membrane turnover of ErbB3.

1. Introduction

The EGF receptor (EGFR) family consists of 4 receptor tyrosine kinases, EGFR/ErbB1, ErbB2/Her2/Neu, ErbB3 and ErbB4. All ErbB proteins have a similar structure, but behave differently with respect to ligand-induced activation and down-regulation [reviewed in 1,2,3]. ErbB3 binds ligands of the heregulin (HRG) family [2,4], but has very weak kinase activity and depends on heterodimerization with other ErbB family members for efficient signal transduction.

Internalization of ErbB proteins is important for regulation of the signal transduction. Upon internalization the fate of the receptor is decided: it is either recycled to the plasma membrane or degraded in lysosomes. We have recently demonstrated that ErbB3 is internalized constitutively by clathrin-mediated endocytosis [5]. Recruitment of clathrin to the plasma membrane is mediated by adaptor protein complexes and accessory proteins which bind either directly to the plasma membrane or to the endocytic cargo. Adaptor protein complex 2 (AP-2) was the first identified and remains the best characterized adaptor protein complex in clathrin-mediated endocytosis. AP-2 consists of four subunits, α1/α2, β, μ2, and σ2 [6], and binds to phosphatidylinositol-(4,5)-bisphosphate (PIP_2) in the plasma membrane, to clathrin and to cargo. Several cargo sorting motifs are recognized by AP-2, such as the tyrosine based YXXØ motif and the di-leucine [DE]XXXL[LIM] sequence [reviewed in 7]. EGFR contains several AP-2 interacting motifs, however, neither of these appear needed for efficient ligand-induced EGFR internalization. To what extent AP-2 is needed for EGFR internalization is disputed [reviewed in 3]. These and other findings suggest that alternative adaptors work in parallel to AP-2 to promote cargo entry into clathrin-coated pits [8,9], and a wide

Abbreviations: AP-2, adaptor protein complex 2; CHC, clathrin heavy chain; CLASP, clathrin-associated sorting proteins; Dyn2, dynamin 2; ENTH, epsin N-terminal homology; HRG, heregulin; UIM, ubiquitin interacting motif.

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range of proteins termed CLASPs (clathrin-associated sorting proteins) can bind internalization signals which differ from the two mentioned [7,10]. Endocytic adaptors other than AP-2, are usually mono- or dimeric proteins interacting with AP-2, clathrin or the plasma membrane in addition to cargo [11]. One alternative internalization signal is ubiquitin. Adaptors recognize ubiquitin through ubiquitin interacting motifs (UIMs), which are present in proteins like Eps15/Eps15R and epsin [12]. Mammalian cells express three epsin isoforms, epsins 1, 2 and 3, and based on the knock-down of all three, the epsins were recently reported to be important coordinators of clathrin-mediated endocytosis in general [13]. Previous studies, however, emphasize epsin 1 as a specific adaptor for internalization of ubiquitinated cargo, such as EGFR and ErbB2 [14–16].

A previous study showed that the cytoplasmic tail of ErbB3 does not interact with AP-2 [17], indicating that adaptor proteins other than, or in addition to, AP-2 are needed for clathrin-dependent endocytosis of ErbB3. To identify ErbB3 interacting adaptors, we knocked down 29 adaptor proteins known to be involved in clathrin-mediated endocytosis and studied the effect on constitutive ErbB3 internalization. Out of the 29 candidates, only knock-down of epsin 1 turned out to inhibit ErbB3 internalization to almost the same degree as knock-down of clathrin heavy chain (CHC). We thus focused on investigation of epsin 1 as an adaptor for constitutive, clathrin-mediated endocytosis of ErbB3.

2. Materials and methods

2.1. Materials

HRG-β1 extracellular domain (HRG-β1 ECD) was from R&D Systems (Minneapolis, MN). Unless otherwise noted, all materials were from Sigma-Aldrich (St. Louis, MO).

2.2. Antibodies

Mouse anti-ErbB3 antibody (clone H3.90.6) was from Lifespan Bioscience, Inc. (Seattle, WA) (LS-C87996) and Thermo Fisher Scientific (Fremont, CA) (Ab-4/H3.90.6/Ab-90). Mouse anti-ErbB3 (Ab-6, clone 2B5) antibody was from Thermo Fisher Scientific. Rabbit anti-epsin 1 (H-130), rabbit anti-dynamin 2 (Dyn2) (H-300), and goat anti-early endosome antigen 1 (EEA1) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse anti-CHC antibody was from BD Biosciences (Erembodegem, Belgium). Mouse anti-GFP (11814 460001) antibody was from Roche (Mannheim, Germany). Rabbit anti-β-tubulin antibody was from Abcam (Cambridge, UK). Alexa Fluor 555-conjugated donkey anti-mouse IgG and Alexa Fluor 647-conjugated donkey anti-goat IgG antibodies were from Life Technologies (Carlsbad, CA). Peroxidase-conjugated donkey anti-rabbit and anti-mouse IgG antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

2.3. Cell culture and treatment

MCF-7 cells and HeLa cells were grown in DMEM with Ultraglutamine I and 4.5 g/l Glucose (Lonza, Basel, Switzerland). A mixture of 50 units of Potassium Penicillin and 50 μg of Streptomycin Sulfate per 1 ml of culture media (Lonza) and 10% (*v*/*v*) FBS were routinely used.

2.4. siRNA library

The siRNA library (OnTARGETplus, custom made by GE Healthcare, Buckinghamshire, UK) containing 4 individual sequences targeting 29 target proteins (Supplementary Table 1A) and CHC (Supplementary Table 1B) was designed based on a recent review [18]. Non-targeting siRNAs: #1 (D-001810-01), #2 (D-001810-02), #3 (D-001810-03), #4 (D-001810-04) and siRNAs targeting Cyclophilin B (D-001820-01) and GAPDH (D-001830-01) (GE Healthcare) were used as negative controls. To knock-down Dyn2, four previously described [19] siRNA sequences (Life Technologies) were used. See Supplementary Table 1B for an overview of all control siRNAs used.

2.5. siRNA transfection of cells

Cells were seeded one day prior to transfection with siRNA using Lipofectamine® RNAiMax. The transfection was performed according to manufacturer's protocol using a final concentration of 50 nM siRNA and the cells were incubated for 72 h before analysis. All siRNA sequences used were from the siRNA library.

2.6. Antibody-based internalization assay

Cells were incubated with a mouse anti-ErbB3 antibody (clone H3.90.6) in MEM with 0.1% bovine serum albumin (BSA) for 30 min on ice, washed and chased in MEM containing 0.1% BSA for 15 min at 37 °C. Before fixation in 10% Neutral Buffered Formalin (4% *w*/*v* formaldehyde), cells were washed for 2 min on ice in low pH buffer (0.5% acetic acid, 0.5 M NaCl, pH 2.8) to remove cell surface bound antibodies. Upon quenching with NH_4Cl and permeabilization with 0.1% Triton X-100 in PBS, internalized anti-ErbB3 antibodies were detected using an Alexa Fluor 555-conjugated anti-mouse antibody. Nuclei were stained with 2 μg/ml of Hoechst 33342.

2.7. High-throughput siRNA screening protocol

The siRNA screen was carried out 3 times, with 3 parallels in each experiment. Each plate contained both positive and negative controls in addition to siRNAs targeting housekeeping genes. One day prior to transfection, MCF-7 cells were seeded on flat plastic-bottomed 96-well plates (NUNC, Thermo Fisher Scientific) coated with 15 μg/ml of Fibronectin. After 72 h incubation with siRNAs cells were subjected to the antibody-based ErbB3 internalization assay (Section 2.6.).

Olympus ScanR system (Olympus SIS, Munster, Germany) with an UPLSAPO 40×/0.95 objective was used for imaging. 49 images were automatically taken from different areas within each well, with identical settings and below pixel saturation. The analysis of internalized anti-ErbB3 positive spots was performed using Olympus ScanR analysis software. In brief, cells were detected based on Hoechst staining (nuclei), the number of spots representing internalized anti-ErbB3 was detected and used to calculate the average internalization of ErbB3 within each cell. Between 500 and 2000 cells were analyzed per target in each experiment.

2.8. Statistical analysis

The whole siRNA library was divided into two plates (A and B). The data were collected from three individual experiments with three parallel plates with the same siRNAs in each experiment. A normalization of the response values was necessary due to a five-fold difference between plates in average number of ErbB3 spots. The normalization was carried out by first multiplying up/down all well values in each plate with a number such that the average across the plate became 1.0. This normalized the responses within screens from plates A and B since all plates within each type are equal in terms of tested sequences. Averages for each well was then computed, and these were normalized between plates type A and B by utilizing the 16 control wells that are found both on A and B (multiplying up/down to get equal averages over the control wells in types A and B). Finally, log2 values of each sequence average and the pooled target averages were calculated. GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA) was used to present the screening results as charts.