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Endosomal trafficking of the ligated FcεRI receptor

Gul'nar V. Fattakhova[a,1], Madhan Masilamani[a,2], Sriram Narayanan[a], Francisco Borrego[a], Alasdair M. Gilfillan[b], Dean D. Metcalfe[b], John E. Coligan[a,*]

[a] Receptor Cell Biology Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852, United States
[b] Mast Cell Biology Section, Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, United States

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ABSTRACT

In addition to initiating signaling cascades leading to mast cell mediator release, aggregation of the high affinity IgE receptor (FcεRI) leads to rapid internalization of the cross-linked receptor. However, little is known about the trafficking of the internalized FcεRI. Here we demonstrate that in RBL-2H3 cells, aggregated FcεRI appears in the early endosomal antigen 1 (EEA1$^+$) domains of the early endosomes within 15 min after ligation. Minimal co-localization of FcεRI with Rab5 was observed by 30 min, followed by its appearance in the Rab7$^+$ late endosomes and lysosomes at later time points. During endosomal sorting, FcεRIα and γ subunits remain associated. In Syk-deficient RBL-2H3 cells, the rate of transport to lysosomes is markedly increased. Taken together, our data demonstrate time-dependent sorting of aggregated FcεRI within the endosomal–lysosomal network, and that Syk may play an essential role in regulating the trafficking and retention of FcεRI in endosomes.

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1. Introduction

The high affinity IgE receptor (FcεRI) is composed of an IgE-binding α chain, a four transmembrane-spanning β subunit and two identical disulfide-linked γ subunits (Kraft and Kinet, 2007). The aggregation of FcεRI on mast cells initiates a biochemical cascade that results in the release of inflammatory mediators. Following ligation, the receptor is rapidly internalized by either clathrin-dependent (Wilson et al., 2004) or clathrin-independent, dynamin-dependent mechanisms (Fattakhova et al., 2006). Despite the fact that FcεRI-mediated signaling in mast cells has been extensively studied (Gilfillan and Tkaczyk, 2006; Rivera and Olivera, 2007), the intracellular trafficking of the receptor and its relation to signaling have not been systematically investigated.

Surface receptors are endocytosed, following the binding of ligand, by a variety of potential endocytic routes (Mayor and Pagano, 2007). Electron microscopy studies have revealed that ligated FcεRI accumulates in transferrin-positive endosomal compartments (Asai et al., 2000; Oliver et al., 2007; Xue et al., 2007), and after time, localizes to structures with properties of lysosomes (Oliver et al., 2007). In addition, studies have suggested that aggregated FcεRI is endocytosed via clathrin-coated pits (Wilson et al., 2004). Our previous study (Fattakhova et al., 2006), however, revealed that, following translocation to detergent-resistant membrane fractions (conceptually termed lipid rafts), the cross-linked FcεRI remains associated with these microdomains upon internalization. Furthermore, in contrast to the aforementioned morphological studies, our data suggested that internalization of cross-linked FcεRI does not require the AP-2/clathrin complex but is dynamin-dependent.

The generalized current view of endocytosis is that intracellular vesicular traffic of internalized surface receptors is mediated by membrane fusion between receptor-containing vesicles and endocytic compartment organelles (Zerial and McBride, 2001). Each fusion step appears to be regulated by Rab proteins and phosphoinositides, generated by the action of phosphoinositide 3-kinase (PI3K). The endocytic pathway can be dissected into distinct Rab-specific compartments: the Rab5$^+$ early endosomal compartment, early/sorting endosomes (Rab4$^+$), recycling endosomes (Rab11$^+$) and the Rab7$^+$ late endosomes. Degradation of internalized receptor complexes usually occurs in LAMP-1$^+$ lysosomes (Markgraf et al., 2007). After internalization from the plasma membrane, proteins first enter early endosomal antigen 1 (EEA1$^+$) early endosomes (Woodman, 2000), not all of which are Rab5$^+$ (Lakadamyali et al., 2006). Thereafter, they traffic according to their fate within the endosomal network described above.

Certain surface receptors, such as the transferrin receptor, are delivered predominantly to the Rab4$^+$, Rab11$^+$ endocytic recycling

* Corresponding author. Tel.: +1 301 496 8247; fax: +1 301 480 2818.
E-mail address: jcoligan@niaid.nih.gov (J.E. Coligan).
[1] Present address: Borstel Research Center, Parkalee 22-26, D-23845 Borstel, Germany.
[2] Present address: Mount Sinai School of Medicine, Division of Pediatric Allergy & Immunology, Department of Pediatrics, One Gustave L Levy Place, New York, NY 10029, United States.

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compartment from where they can recycle back to the cell surface (Maxfield and McGraw, 2004). Ligation of many other surface receptors, such as the T cell receptor (TCR), predominantly results in receptor clustering that is followed by down-regulation through endocytosis and, subsequently, proteosomal and lysosomal degradation (Geisler, 2004). Rapid degradation serves to attenuate signaling via removal of activated receptor complexes. The process of endocytosis may also serve to regulate signaling pathways required for transcriptional regulation (Kapp-Barnea et al., 2006).

In this study, we examine the endocytic trafficking of internalized ligated FcεRI using confocal microscopy. We show that aggregated FcεRI first localizes to EEA1$^+$ early endosomes, and minimally co-localizes with Rab5$^+$ structures. Rather than trafficking via Rab4$^+$ and Rab11$^+$ endosomal compartments, FcεRI appears to ultimately traffic through the Rab7$^+$ late endosomes and LAMP-1$^+$ lysosomes in a time-dependent manner. The FcεRIα and -γ chains remain associated during trafficking. In Syk-deficient cells, the rate of FcεRI migration to lysosomes is markedly enhanced, suggesting that Syk may play a role in modulating receptor traffic.

2. Materials and methods

2.1. Reagents and cell lines

Antibodies and reagents used in this study were obtained from the following vendors: streptavidin AlexaFluor 405, goat-anti-mouse IgG conjugated to AlexaFluor 594, goat-anti-rabbit IgG conjugated to AlexaFluor 647 or AlexaFluor 594, AlexaFluor labeling kits, and cell culture reagents were from Invitrogen Inc. (Carlsbad, CA); anti-DNP-specific mouse IgE clone SPE-7 mAb, dinitrophenyl-conjugated human serum albumin (DNP-HSA), biotinamidohexanoic acid *N*-hydroxysuccinimide were from Sigma (St. Louis, MO); rabbit polyclonal anti-FcR γ subunit antisera was purchased from Upstate Biotechnology (Lake Placid, NY); FITC-labeled rat anti-mouse IgE monoclonal antibody (mAb) and purified anti-EEA1 mAb were purchased from BD Biosciences (San Jose, CA); the LAMP-1 mAb used for confocal microscopy originated from J.T. August and J.E.K. Hildreth and was from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA, USA. Biotinylation of IgE was carried out as described (Cole et al., 1987). AlexaFluor 488 conjugates of anti-DNP IgE were prepared according to the manufacturer's recommendations (Invitrogen Inc.). The wild-type EGFP-tagged Rab5 constructs were a kind gift from Dr. Juan Bonifacino (NICHD/NIH, Bethesda, MD). GFP constructs of Rab4, Rab7, and Rab11 were kind gifts from Dr. Marino Zerial, Max Plank Institute of Molecular and Cell Biology, Dresden, Germany.

The parental rat basophilic leukemia RBL-2H3 cell line and Syk-deficient RBL-2H3 cells, kindly provided by Dr. Reuben Siraganian (NIDCR/NIH), were cultured as monolayers in ISCOVE's medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM non-essential amino acids (all from Biosource International, Camarillo, CA), and 5 μg/ml plasmocin (InvivoGen, San Diego, CA).

2.2. Analysis of surface expression and internalization by flow cytometry

Analyses of FcεRI cell surface expression and receptor internalization were performed as described (Saitoh et al., 2003; Fattakhova et al., 2006). Adherent RBL-2H3 cells were incubated with 300 ng/ml anti-DNP mouse IgE mAb for 18 h at 37 °C in culture medium. The cells were harvested, and the unbound IgE was washed away, followed by resuspension in Tyrode buffer (10 mM HEPES pH 7.4, 130 mM NaCl, 2.7 mM KCl, 0.4 mM Na_2HPO_4, 1.8 mM $CaCl_2$, 1.3 mM $MgSO_4$, 5.6 mM glucose) at 4×10^5 cells/ml. FcεRI was cross-linked by adding 250 ng/ml DNP-HSA for the indicated lengths of time. Internalization was stopped by placing cells at 4 °C and washing with ice-cold PBS. Cells were blocked with 5% rat serum for 30 min at 4 °C on ice to prevent non-specific staining with secondary Ab and then incubated with FITC-labeled rat anti-mouse IgE for 30 min on ice. For the analysis of total (surface plus intracellular) IgE, cells were fixed with 2.5% paraformaldehyde for 20 min at room temperature and incubated with FITC-labeled rat anti-mouse IgE in permeabilizing buffer (0.1% saponin, 1 mM KCl, 1 mM $MgSO_4$ in PBS). Inhibition of Syk activity by piceatannol was done as described (Lauvrak et al., 2006; Oliver et al., 1994). Briefly, IgE-sensitized cells ($3–5 \times 10^5$ ml^{-1}) were incubated in the presence of 50 μg/ml piceatannol added in DMSO (5 μl of a 10 mg/ml stock solution per ml of culture medium) or the same amount of DMSO alone for 1 h prior to antigen stimulation. Flow cytometry was performed on a FACSORT™, and the data were analyzed with FLOWJO™ (Tree Star, Inc., Ashland, OR, USA) or CELLQUEST™ software (BD Biosciences).

2.3. Immunostaining, confocal microscopy, image acquisition and analysis

RBL-2H3 cells were seeded at 7×10^5 cells/ml on glass cover slips (Thomas Scientific, Swedesboro, NJ) in 24-well plates (Corning Costar, Rochester, NY) and cultured in serum-free medium containing IgE-AlexaFluor 488 for 18 h. For analysis of Rab4, Rab5, Rab7 or Rab11 co-localization, cells were transfected with the appropriate EGFP-tagged construct, and sensitized with biotinylated IgE 24 h after transfection. During sensitization, IgE conjugates stain only surface FcεRIα chains in intact living cells. After 16 h, the cells were washed three times with serum-free medium, 400 μl of colorless OptiMEM cell medium (Invitrogen Inc.) was added to each well, and cells were incubated in the presence of 100 ng/ml of DNP-HSA for the indicated periods of time at 37 °C. Cells were then washed three times with ice-cold PBS and blocked with 5% rat serum in PBS for 30 min on ice. After extensive washing, cells were fixed with 3.7% paraformaldehyde in PBS for 15 min at 37 °C, then permeabilized with 0.1% Triton X-100/PBS. γ chain staining was made in fixed and permeabilized cells, as the rabbit anti-γ antibodies are specific for the cytoplasmic region of γ subunits (Jouvin et al., 1995; Maurer et al., 1996; Repetto et al., 1996). EEA1 and LAMP-1 were visualized using the appropriate primary and secondary antibodies. All incubations with unlabeled antibodies were done at room temperature for 40 min with gentle shaking, then, the cells were washed three times with permeabilization buffer and stained with the appropriate AlexaFluor 594 or AlexaFluor 647 conjugated secondary antibodies. Streptavidin AlexaFluor 405 was used to visualize biotinylated IgE.

All images were collected on a Leica TCS SP2 AOBS microscope (Leica Microsystems, Heidelberg GmbH, Mannheim, Germany) at the Biological Imaging Facility (Research Technologies Branch, NIAID, NIH, Bethesda). The images were acquired using an oil immersion 63× objective, NA 1.32 in sequential mode. The protocol for image acquisitions and a combination of lasers and intensities were set appropriately to avoid cross-talk between dyes. Image analysis was done using a Leica Confocal Software version 2.5, build 1104 (Leica Microsystems), Imaris version 5.7.1 (Bitplane AG) and by Adobe Photoshop version 7.0 (Adobe Systems). Movies were made from 3D animation of representative cells constructed from confocal images collected in *Z* plane (20–30 per section) and animated at 15 frames/s.

Three-dimensional (3D) images of representative cells acquired in *Z*-axis stacks (0.3 μm) were analyzed for co-localization using