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Research Article

Expression and subcellular localization of the Qa-SNARE syntaxin17 in human eosinophils

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ABSTRACT

Background: SNARE members mediate membrane fusion during intracellular trafficking underlying innate and adaptive immune responses by different cells. However, little is known about the expression and function of these proteins in human eosinophils, cells involved in allergic, inflammatory and immunoregulatory responses. Here, we investigate the expression and distribution of the Qa-SNARE syntaxin17 (STX17) within human eosinophils isolated from the peripheral blood.
Methods: Flow cytometry and a pre-embedding immunonanogold electron microscopy (EM) technique that combines optimal epitope preservation and secondary Fab-fragments of antibodies linked to 1.4 nm gold particles for optimal access to microdomains, were used to investigate STX17.
Results: STX17 was detected within unstimulated eosinophils. Immunogold EM revealed STX17 on secretory granules and on granule-derived vesiculotubular transport carriers (Eosinophil Sombrero Vesicles-EoSVs). Quantitative EM analyses showed that 77.7% of the granules were positive for STX17 with a mean ± SEM of 3.9 ± 0.2 gold particles/granule. Labeling was present on both granule outer membranes and matrices while EoSVs showed clear membrane-associated labeling. STX17 was also present in secretory granules in eosinophils stimulated with the cytokine tumor necrosis factor alpha (TNF-α) or the CC-chemokine ligand 11 CCL11 (eotaxin-1), stimuli that induce eosinophil degranulation. The number of secretory granules labeled for STX17 was significantly higher in CCL11 compared with the unstimulated group. The level of cell labeling did not change when unstimulated cells were compared with TNF-α-stimulated eosinophils.
Conclusions: The present study clearly shows by immunanonogold EM that STX17 is localized in eosinophil secretory granules and transport vesicles and might be involved in the transport of granule-derived cargos.

1. Introduction

Secretion is an essential biological activity of all eukaryotic cells by which they release specific products in the extracellular space during physiological and pathological events. In cells from the immune system, such as eosinophils, basophils, neutrophils and macrophages, secretory mechanisms underlie the functions of these cells during allergic, inflammatory and immunoregulatory responses (reviewed in [1,2]).

Our Group has been studying mechanisms of intracellular trafficking and secretion in human eosinophils [3–7]. Eosinophil responses involve secretion of distinct cationic proteins and numerous cytokines with multiple functional activities. These mediators are released in a tightly orchestrated manner to regulate the progression of immune responses (reviewed in [8–10]). Different from lymphocytes that must exclusively synthesize proteins prior to secretion and similar to neutrophils [11] and mast cells [12], both cationic proteins and cytokines are additionally stored as preformed pools within eosinophil secretory granules [13].

In human eosinophils, vesicle-mediated transport of proteins from secretory granules is commonly described both *in vitro* and *in vivo* during different conditions, including inflammatory and allergic disorders [14–20]. Large carriers, identified as

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vesiculotubular structures of complex plasticity, termed Eosinophil Sombrero Vesicles (EoSVs), in addition to small vesicles, participate in the vesicular trafficking of eosinophil granule-stored mediators, such as IL-4 [3,4] and major basic protein (MBP) [7]. EoSVs are constantly found in biopsies of patients with inflammatory diseases such as eosinophilic esophagitis [20] and bowel disease [8].

The volume and complexity of vesicular traffic in eosinophils and other cells from the immune system require a selective machinery to ensure the accurate docking and fusion of carrier vesicles at their designated target membranes. SNARE proteins (*N*-ethylmaleimide sensitive factor attachment protein receptors) that are present on secretory granule and plasma membranes likely mediate this fusion.

SNAREs are generally small (14–40 kDa), coiled-coil forming proteins that are anchored to the membrane via a C-terminal anchor. They were originally classified as v- (vesicle-associated) or t- (target-membrane) SNAREs, on the basis of their locations and functional roles in a typical trafficking step. However, this orientation is not always maintained and an alternative structure-based terminology has now been used, wherein the family is divided into R-SNAREs and Q-SNAREs, on the basis of whether the central functional residue in their SNARE motif is arginine (R) or glutamine (Q). Q-SNAREs are then further classified into Qa, Qb, Qc and Qb,c subtypes based on where their SNARE domain(s) would sit in an assembled trans-SNARE complex (reviewed in [1,21]).

So far, few studies have documented SNAREs at subcellular sites of human eosinophils. Only three SNAREs, all R-SNAREs members, were characterized in intracellular locations: the vesicle-associated membrane protein (VAMP)2, found predominantly in vesicles [22–24], and VAMP7 and VAMP8, which were documented in granule-enriched fractions [25].

Here, we investigate the expression and subcellular localization of the Qa-SNARE syntaxin17 (STX17) within human eosinophils. By using flow cytometry and an immunonanogold electron microscopy technique that combines different strategies for optimal labeling and morphology preservation [26], we provide the first identification of STX17 in human eosinophils. This SNARE is localized in eosinophil secretory granules and EoSVs from both unstimulated and stimulated eosinophils and might be involved in the transport of granule-derived specific cargos.

2. Material and methods

2.1. Eosinophil isolation, stimulation and viability

Granulocytes were isolated from the blood of different healthy donors. Eosinophils were enriched and purified by negative selection using human eosinophil enrichment cocktail (StemSep™, StemCell Technologies, Seattle WA, USA) and the MACS bead procedure (Miltenyi Biotec, Auburn, CA, USA), as described [7], with the exception that hypotonic red blood cell (RBC) lysis was omitted to avoid any potential for RBC lysis to affect eosinophil function. Eosinophil viability and purity were greater than 99% as determined by ethidium bromide (Molecular Probes, OR, USA) incorporation and cytocentrifuged smears stained with HEMA 3 stain kit (Fisher Scientific, TX, USA), respectively. Experiments were approved by the Beth Israel Deaconess Medical Center Committee on Clinical Investigation, and informed consent was obtained from all subjects. Purified eosinophils (10^6 cells/mL) were stimulated with TNF-α (200 ng/mL; R&D Systems, USA) or recombinant human CCL11 (eotaxin-1) (100 ng/mL; R&D Systems, Minneapolis, MN) in RPMI-1640 medium plus 0.1% ovalbumin (OVA) (Sigma, St. Louis, MO, USA), or medium alone at 37 °C, for 1 h as before [27].

2.2. Antibody reagents

Antibodies for STX17 detection in eosinophils were an affinity-purified goat polyclonal antibody raised against a peptide mapping within a cytoplasmic domain of STX17 of human origin (Santa Cruz Biotechnology, TX, USA, sc-107095) used in parallel with control goat IgG (Santa Cruz Biotechnology) at concentrations of 5 μg/mL (immunoEM) or 10 μg/mL (flow cytometry). Secondary antibody for immunoEM studies was an affinity-purified rabbit anti-goat Fab fragment conjugated to 1.4-nm gold particles (1:100, Nanogold®, cat. # 2006, Nanoprobes; Stony Brook, NY). Secondary antibodies for flow cytometry were anti-goat antibodies conjugated to FITC (10 μg/mL, Jackson ImmunoResearch laboratories Inc., West Grove, PL, USA).

2.3. Conventional TEM

For conventional TEM, isolated eosinophils were fixed in a mixture of freshly prepared aldehydes (1% paraformaldehyde and 1.25% glutaraldehyde) in 1 M sodium cacodylate buffer for 1 h at room temperature (RT), embedded in 2% agar [19] and kept at 4 °C for further processing. Agar pellets containing eosinophils were processed as described. Briefly, samples were post-fixed in 1% osmium tetroxide in Sym-Collidine buffer, pH 7.4, for 2 h at RT. After washing with sodium maleate buffer, pH 5.2, they were stained en bloc in 2% uranyl acetate in 0.05 M sodium maleate buffer, pH 6.0 for 2 h at RT and washed in the same buffer as before prior to dehydration in graded ethanols and infiltration and embedding with a propylene oxide-Epon sequence (Eponate 12 Resin; Ted Pella, Redding, CA, USA) [19]. Specimens were examined using a transmission electron microscope (CM 10, Philips) at 60 kV.

2.4. Cell preparation for immunonanogold EM

For immunoEM, purified eosinophils were immediately fixed in fresh 4% paraformaldehyde in 0.02 M phosphate-buffered saline (0.15 M NaCl) (PBS), pH 7.4 [26]. Cells were fixed for 30 min at room temperature (RT), washed in PBS and centrifuged at 1500*g* for 1 min. Samples were then resuspended in molten 2% agar in PBS and quickly recentrifuged. Pellets were immersed in 30% sucrose in PBS overnight at 4 °C, embedded in OCT compound (Miles, Elkhart, IN, USA), and stored in −180 °C liquid nitrogen for subsequent use.

2.5. Pre-embedding immunonanogold EM

Pre-embedding immunolabeling was carried out before standard EM processing (postfixation, dehydration, infiltration, resin embedding and resin sectioning). Immunonanogold was performed on cryostat 10 μm sections mounted on glass slides. After testing different section thicknesses, we found that 10 μm enabled optimal penetration of the antibodies [26]. All labeling steps were carried out at RT as before [26] as follows: (a) one wash in 0.02 M PBS, pH 7.6, 5 min; (b) immersion in 50 mM glycine in 0.02 M PBS, pH 7.4, 10 min; (c) incubation in a mixture of PBS and bovine serum albumin (PBS–BSA buffer; 0.02 M PBS plus 1% BSA) containing 0.1% gelatin (20 min) followed by PBS–BSA plus 10% normal goat serum (NGS) (30 min). This step is crucial to block non-specific binding sites; (d) incubation with primary antibody (1 h); (e) blocking with PBS–BSA plus NGS (30 min); (f) incubation with secondary antibody (1 h); (g) washing in PBS–BSA (three times of 5 min each); (h) postfixation in 1% glutaraldehyde (10 min); (i) five washings in distilled water; (j) incubation with HQ silver enhancement solution in a dark room according to the manufacturer's instructions (Nanoprobes) (10 min). This step enables a nucleation of silver ions around gold particles. These ions