Characterization of Mast Cell Secretory Granules and Their Cell Biology

Nurit Pereg Azouz,^{1,*} Ilan Hammel,² and Ronit Sagi-Eisenberg¹

Exocytosis and secretion of secretory granule (SG) contained inflammatory mediators is the primary mechanism by which mast cells exert their protective immune responses in host defense, as well as their pathological functions in allergic reactions and anaphylaxis. Despite their central role in mast cell function, the molecular mechanisms underlying the biogenesis and secretion of mast cell SGs remain largely unresolved. Early studies have established the lysosomal nature of the mast cell SGs and implicated SG homotypic fusion as an important step occurring during both their biogenesis and compound secretion. However, the molecular mechanisms that account for key features of this process largely remain to be defined. A novel high-resolution imaging based methodology allowed us to screen Rab GTPases for their phenotypic and functional impact and identify Rab networks that regulate mast cell secretion. This screen has identified Rab5 as a novel regulator of homotypic fusion of the mast cell SGs that thereby regulates their size and cargo composition.

Mast Cells

MAST CELLS ARE BONE marrow-derived cells that traverse the vascular space and enter the tissues, where they complete their differentiation and maturation process, localizing predominantly within connective tissues and epithelial surfaces (Metcalf *et al.*, 1997). These granulated cells of the immune system are best known for their involvement in allergic reactions. Binding of allergen-specific immunoglobulin E (IgE) class antibodies to the mast cell FcεRI high-affinity receptor, followed by antigen-induced receptor aggregation, initiates a signaling cascade that culminates in the fusion of the secretory granules (SGs) with the plasma membrane (PM) leading to the discharge of their contents [reviewed in Rivera and Gilfillan (2006)]. As a result of this degranulation, multiple inflammatory mediators are secreted. The latter includes mast cell proteases that increase tissue permeability facilitating the infiltration of immune cells and vasoactive amines such as histamine and serotonin (in rodents) that increase vascular permeability and stimulate smooth muscle contraction (Schwartz and Austen, 1980; Gordon and Galli, 1990). This initial event of degranulation is followed by the *de novo* synthesis and secretion of a large array of biologically potent substances, including arachidonic acid metabolites, multiple cytokines, and chemokines (Gordon and Galli, 1990; Gordon *et al.*, 1990; Metz and Maurer, 2007), which collectively with the preformed mediators initiate early and late inflammatory responses. Mast cells are also activated, independently of

IgE, by neuropeptides (Lagunoff *et al.*, 1983), toxins (Depinay *et al.*, 2006), bacterial and viral antigens (Abel *et al.*, 2011; Avila and Gonzalez-Espinosa, 2011), immune cells (Mekori and Metcalfe, 1999; Minai-Fleminger *et al.*, 2010), as well as by many of their own secreted mediators, which further amplify the inflammatory response (Gilfillan *et al.*, 2009; Rudich *et al.*, 2012). While these inflammatory responses are recognized as allergy in hypersensitive individuals, physiologically, they mediate mast cell functions in innate and adaptive immunity (Shelburne and Abraham, 2011; Tsai *et al.*, 2011).

Mast Cell Exocytosis

The mechanisms by which mast cells secrete their *de novo* synthesized mediators still need to be resolved. The mechanisms by which they secrete their SG contents are better characterized, although the pathways and molecular entities involved remain poorly understood. Best characterized is IgE-dependent exocytosis, whose stimulus-secretion coupling networks have been delineated (Benhamou and Blank, 2010; Rivera *et al.*, 2008). The latter may involve full exocytosis, whereby fusion of PM docked SGs with the PM allows complete expulsion of their contents, or kiss-and-run fusion that partially releases the SG cargo through a relative narrow and transient fusion pore. Depending on the strength of the signal, the efficacy of exocytosis can be significantly increased by compound secretion, which involves homotypic fusion of the SGs thus allowing rapid

Departments of ¹Cell and Developmental Biology and ²Pathology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel.

*Present Address: Division of Allergy and Immunology, Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, Ohio.

discharge of SGs located distally bypassing the need for their transport to the PM (Deng *et al.*, 2009; Blank, 2011; Cohen *et al.*, 2012). Indeed, EM analyses (Anderson *et al.*, 1973) combined with membrane capacitance measurements (Alvarez de Toledo and Fernandez, 1990a, 1990b) have identified homotypic fusion of the SGs as an integral part of the exocytic process. Both sequential and multivesicular modes of compound exocytosis were noted indicating that homotypic fusion takes place after the initial fusion of SGs with the PM, but prefused SGs may exist as well. Consistent with the complexity of mast cell exocytosis, multiple SNARE proteins have been implicated in playing a role in mast cell exocytosis. The latter includes VAMP 2, 3, 4, 7, and 8, SNAP23 and Syntaxins 3, 4, and 6 (Puri and Roche, 2008; Sander *et al.*, 2008; Tiwari *et al.*, 2008; Lorentz *et al.*, 2012; Woska and Gillespie, 2012; Brochetta *et al.*, 2014).

The SGs of Mast Cells

The SGs of mast cells display lysosomal features. Thus, in addition to their specialized cargo of inflammatory mediators, such as histamine, the SGs contain lysosomal enzymes (Schwartz and Austen, 1980) and lysosomal membrane proteins (Suarez, 1987), have an acidic pH (Johnson *et al.*, 1980; Lagunoff and Rickard, 1983), receive and exocytose endocytic cargo in a regulated manner (Cohen *et al.*, 2012), recycle SG proteins (Bonifacino *et al.*, 1989), and are regulated by endocytic recycling controlling synaptotagmins (Grimberg *et al.*, 2003; Haberman *et al.*, 2007). Therefore, mast cell SGs are regarded as lysosome-related organelles (LROs) or secretory lysosomes. However, unlike natural killer cells or cytotoxic T lymphocytes in which LROs arise from lysosomes, mast cells seem to contain both conventional lysosomes and SGs that possess lysosomal activity (Schwartz and Austen, 1980). Indeed, normal SGs alongside abnormal lysosomes were detected in mast cells in biopsies derived from lysosomal storage disease patients (Hammel *et al.*, 1993a), and the fractionation analyses demonstrated the distribution of lysosomal β -hexosaminidase between histamine-containing SGs and histamine-free fractions, which most likely correspond to the conventional lysosomes (Baram *et al.*, 1999; Grimberg *et al.*, 2003; Haberman *et al.*, 2007).

Strikingly, despite their central role in mast cell function under both physiological (i.e., host defense) and pathological (i.e., allergy) conditions, the molecular mechanisms underlying the biogenesis of the mast cell SGs remain largely unresolved. Morphometric findings derived from EM studies of mast cells supported a model, in which fusion between SGs occurs not only during their compound exocytosis in triggered cells but is also a key post-Golgi mechanism for generating mature SGs. According to this model (Fig. 1), newly formed unit granules (UGs) homotypically fuse forming granules whose volumes are multiples of the UGs' volume (Hammel *et al.*, 2010). The so-formed immature granule (IG) can then further fuse with another IG or with a mature SG (Fig. 1). A clear advantage of such a mechanism is that SGs larger than UGs are formed that can store considerable amounts of secretory cargo that is ready to go during degranulation; yet, fusion with UGs allows the incorporation of new updated cargo formed specifically to address new environmental demands (Hammel and

FIG. 1. Model for Rab5-mediated fusion of mast cell secretory granules (SGs). According to this model, unit granules exit the Golgi aggregate (numbers indicate equivalent progranule size) and undergo Rab5-mediated homotypic fusion (step a) creating immature secretory granules (IGs). The latter can further fuse with other IGs before their maturation into SGs, a process mediated by cargo removal and dissociation of Rab5 (step b). Rab5-bound IGs can fuse with other IGs or with a mature SG or with an endosome formed in a Rab5-dependent manner (steps I–III). This latter step allows integration of endocytic cargo into the SG.

Meilijson, 2012). However, while the morphological analyses support this model firmly, the mechanism underlying the SG homotypic fusion during biogenesis remained elusive. Who controls this process? Which SNAREs mediate the fusion process? How do the SGs acquire endocytic cargo and lysosomal features? How do they mature? How do they acquire exocytosis competence? How do they move to the PM? What dictates which mode of exocytosis will eventually take place? What are the core components of the fusion machinery? These questions remain open.

Rab GTPases and Mast Cell Exocytosis

To begin addressing the unresolved questions of mast cell exocytosis, we have recently developed a novel high-resolution imaging-based methodology that allows functional genomics analyses of mast cell exocytosis. Our methodology is based on cotransfections of a gene of interest, fused to GFP, and Neuropeptide Y (NPY) fused to the monomeric red fluorescent protein (mRFP)-mRFP. The latter (NPY-mRFP) is sorted to the SGs and secreted alongside the

FIG. 2. Constitutively active Rab5A induces giant SGs. Expression of an active mutant of the small GTPase Rab5A in rat basophilic leukemic mast cells results in the formation of giant SGs. Visualized in this picture are SGs that contain Neuropeptide Y-mRFP (magenta), a fluorescent reporter that is targeted to the SGs and serotonin, an endogenous resident of the SGs that is labeled by anti-serotonin antibodies (blue), in control cells (*left panel*) or in cells that express the active GFP-Rab5A mutant (green, *right panel*). Scale bar 2 μ m.

endogenous mediators, thus serving as an appropriate reporter for both the visualization of the SGs and quantification of the exocytic process (Azouz *et al.*, 2012). We used this methodology to screen Rab GTPases, which are master regulators of vesicular trafficking (Fukuda, 2008), for their phenotypic and functional impact on mast cell exocytosis (Azouz *et al.*, 2012). This screen has identified 30 Rabs as potential regulators of exocytosis in rat basophilic leukemic (RBL) cells, a commonly studied mast cell line (Azouz *et al.*, 2012). This list included Rabs (i.e., Rab4A, Rab13, Rab32, Rab37, Rab38) that affected exclusively the IgE-mediated secretion mainly mediated by kiss-and-run exocytosis (Deng *et al.*, 2009), Rabs (i.e., Rab3A, Rab6A, Rab14, Rab17, Rab21, Rab23, Rab28, Rab29, Rab35, Rab39A) that affected selectively the secretion triggered by the combination of a Ca^{2+} ionophore and the phorbol ester TPA (Ion/TPA), which involves full exocytosis (Deng *et al.*, 2009), and Rabs (i.e., Rab7, Rab8A, Rab9, Rab10, Rab11A, Rab12, Rab19, Rab20, Rab22A, Rab27, Rab42, Rab43) that affected both types of secretion triggered by either stimulus (Azouz *et al.*, 2012). Intriguingly, the latter group included Rabs that localize to the SGs and Rabs that are implicated in regulating transport from the endocytic recycling compartment (ERC), a discrete endosomal organelle involved in slow endocytic recycling. Therefore, while the SG localized Rabs might regulate final steps of exocytosis that are shared by kiss-and-run and full exocytosis, the involvement of ERC controlling Rabs implicates transport through the ERC in playing a heretofore unrecognized role in mast cell exocytosis. In addition, noteworthy is the selective involvement of Rabs (i.e., Rab2A, Rab6, Rab14, and Rab39), known to regulate steps along the biosynthetic/secretory pathway, in controlling Ion/TPA-induced secretion, the reasons for which await further investigation.

Rab5 Controls SG Fusion

Among the Rabs that displayed a remarkable phenotypic impact, we identified Rab5 as a regulator of SG fusion (Azouz *et al.*, 2014). Coexpression of constitutively negative GDP-locked mutants of the endogenously expressed isoforms of Rab5 (Rab5A, Rab5B, and Rab5C) or expression of Rab5A/B/C targeting shRNAs, reduced significantly the SGs' size with a concomitant increase in their numbers (Azouz *et al.*, 2014). Conversely, expression of a GTP-locked constitutively active (CA) Rab5A mutant (Rab5A Q79L, herein: CA Rab5A), which is known to facilitate

homotypic fusion of early endosomes (Stenmark *et al.*, 1994), resulted in the formation of giant Rab5A-decorated vesicles, which we identified as SGs based on their content of the secretory cargo NPY-mRFP and serotonin (Fig. 2) and their capacity to exocytose in a regulated manner (Azouz *et al.*, 2014). The inverse relationship between the number and size of SGs suggested that Rab5-mediated homotypic fusion of the SGs. Indeed, high-resolution imaging of the giant SGs by confocal microscopy of living cells, which detected Rab5A decorating fusing SGs, and electron micrographs that depicted considerably larger SGs and homotypic fusion between two or more granules in CA Rab5A-expressing cells, have reinforced this conclusion (Azouz *et al.*, 2014). The interaction of Rab5 with the SGs was transient and occurred shortly after their Golgi exit (Azouz *et al.*, 2014). Therefore, Rab5 transiently and preferably associates with newly formed SGs. This assignment of Rab5 as principal regulator of SG fusion during their biogenesis is highly consistent with our proposed unit addition model (Hammel *et al.*, 1983, 1993b) discussed above (Fig. 1). Hence, assuming that in analogy to its role in endosome fusion, Rab5 is recruiting the fusion machinery, the selective and transient association of Rab5A with newly formed SGs is compatible with a fusogenic apparatus, in which only newly generated unit and immature granules have the capacity to fuse with other SGs (Fig. 1). Furthermore, we could also demonstrate the occurrence of Rab5-mediated heterotypic fusion between SGs and endosomes that mainly serves to integrate cargo that travels through the PM and endosomes, such as CD63, into the SGs (Fig. 1) (Azouz *et al.*, 2014).

Recycling of SGs

While Rab5 controls the SG size by facilitating SG fusion, an alternative mechanism for regulating the SG size is by cargo recycling. Mutations that perturb this process, for example, mutations in the Chediak-Higashi syndrome/lysosomal trafficking regulator (CHS/Lyst) in humans, with the Chediak-Higashi syndrome, and in beige (*Lyst^{bg}/Lyst^{bg}*) mice (Durchfort *et al.*, 2012) result in giant SGs and can cause significant pathology. Indeed, we have shown that the same phenotype of increased sized SGs is caused by knockdown of synaptotagmin 3, a member of the synaptotagmin family of traffic regulating proteins (Grimberg *et al.*, 2003) that regulates endocytic recycling (Grimberg *et al.*,

2003; Masztalerz *et al.*, 2007). Therefore, the ultimate size of the mast cell SG is dictated by the balance between Rab5-mediated SG fusion and recycling regulated by synaptotagmin 3 and the Lyst protein. We hypothesize that by controlling the SG size, mast cells regulate the ratio between SG cargo and the granular exocytic machinery. Therefore, alternations in the SG size might potentially affect the extent and kinetics of exocytosis. Consistent with this concept, the vesicle size was recently shown to play an important role in dictating the mode of exocytosis in lactotrophs (Flaşker *et al.*, 2013). Thus, large prolactin-containing granules secrete their contents by full fusion, as opposed to the smaller vesicles, which displayed transient exocytosis (Flaşker *et al.*, 2013). Whether or not this control mechanism also applies to mast cell exocytosis remains to be determined.

Acknowledgment

This work was supported by a grant from the Israel Science Foundation, founded by the Israel Academy for Sciences (1139/12 to R.S.-E.).

Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Ronit Sagi-Eisenberg, PhD

Department of Cell and Developmental Biology

Sackler Faculty of Medicine

Tel Aviv University

Tel Aviv 69978

Israel

E-mail: histol3@post.tau.ac.il

Received for publication May 29, 2014; accepted May 29, 2014.