Innate immunity and exocytosis of antimicrobial peptides

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In Drosophila, anti-microbial peptides are activated and secreted in response to microbial challenge, but the intracellular route of anti-microbial peptide trafficking and the regulatory mechanism controlling their secretion are yet to be fully characterized. We have demonstrated that in Drosophila immune response cells (i.e., fat body cells and hemocytes) the anti-microbial peptide Drosomycin is localized within Rab4 and Rab11 intracellular vesicles. Moreover, both of these small GTPases were required for the delivery of this Drosomycin cargo to the plasma membrane. At the plasma membrane, exocytosis and Drosomycin secretion depend on the SNARE protein Syntaxin1A. Thus, the depletion of Syntaxin1A impaired the release of this antimicrobial peptide, and resulted in the accumulation of Drosomycin and Rab11 carrier vesicles near the plasma membrane. Intriguingly, a similar phenotype was generated by the loss of the adaptor protein 14–3-3ε; there was accumulation of Rab11 vesicles and Drosomycin containing vesicles near the plasma membrane, and a concomitant increase in the susceptibility of 14–3-3ε mutant Drosophila to acute bacterial infection. This suggested that 14–3-3ε, possibly via interaction with Syntaxin1A, is required to promote exocytosis of immune-mediators, thereby regulating innate immune secretion and organism survival under conditions of immune stress.

Innate immunity forms a crucial first line of defense against microbial challenge. Upon either pathogen or other environmental challenge, cells of the human innate immune system (e.g., neutrophils, eosinophils, mast cells, macrophages, natural killer, basophils) can generate a burst of pro-inflammatory and other immune mediators, which are secreted via the process of vesicular exocytosis.[1] When unchecked, the secretion of these immune mediators can play a central role in debilitating diseases including cancer, asthma, rheumatoid arthritis and atherosclerosis.[2] Despite exocytosis being recognized as an essential step in the propagation of an immunological response, much of the mechanism underlying this critical cellular event remains unclear.[1]

The main immune response in insects involves innate immunity; partly because of this, the past decade has seen a fundamental appreciation of the relevance of insect effectors and regulatory mechanisms, to the understanding of innate immunity. The recognized steps of innate immunity pathways, such as the Toll/IL-1 and IMD/TNF-α pathways, transcription factors of the NF-κB/Rel family, as well as some cytokines (e.g., TNF-α/Eiger) and anti-microbial peptides (AMPs), are highly orthologous between mammals and Drosophila.[3,4] In the Drosophila innate immune system, cells called hemocytes (professional macrophages); and the fat body (humoral response organ, the prototype of the vertebrate liver), both produce anti-microbial peptides. To better understand how the anti-microbial peptide Drosomycin is secreted from fat body cells and hemocytes we have activated the immune response in Drosophila by acute bacterial infection. Following exposure to bacteria, newly synthesized anti-microbial peptides are trafficked to the cell periphery of immune response cells, where they can be released into the extracellular milieu, to

combat infection[5,6] This study showed that Drosomycin was trafficked from the perinuclear region of fat body cells, via an anterograde vesicular trafficking route that involved recycling endosomes, marked by the membrane presence of the guanosine triphosphatase (GTPase) proteins, Rab4 and Rab11. These GTPases function to control vesicular compartments and to target vesicles and their internal cargo to specific cellular destinations, including the plasma membrane.[7] The silencing of Rab4 and Rab11 retained Drosomycin in the perinuclear region, blocking its traffic toward the plasma membrane; and demonstrating that Rab4 and Rab11 are essential for Drosomycin AMP antero-grade trafficking in Drosophila.[8] Once in close proximity to the plasma membrane, vesicles can be tethered and subsequently docked onto the inner surface of the plasma membrane, by vesicle associated proteins of the soluble NSF attachment protein receptor (SNARE) family (e.g., Syntaxins), which regulate membrane fusion and the release of cargo from these exocytic vesicles.[2,9] Drosophila Syntaxin1A, like its vertebrate counterparts,[10-12] appears to function at the final stage of exocy-tosis; as Syntaxin1A depletion by RNAi resulted in reduced secretion of Droso-mycin, along with accumulation of Rab11 and Drosomycin containing vesicles near the plasma membrane of fat body cells.

Yeast-2-hybrid protein interaction studies have recently identified Drosophila Syntaxin1A as a binding partner for the adaptor protein 14-3-3.[13] Moreover, we found a striking similarity between the phenotypes for the loss of Syntaxin1A and 14-3-3, both of which blocked Rab11-mediated exocytosis and secretion of anti-microbial peptides, in immune response tissues.[8] Stimulated by the expo-sure to bacterial pathogens, the secretion of antimicrobial peptides is expected to confer an organism with resistance to this microbial infection, and is therefore critical for host survival. Consistent with a significant role for 14-3-3 in exocytosis and AMP secretion, Drosophila *14-3-3ε* mutants exhibited a compromised innate immune response; exhibiting dramatically reduced survival when challenged by acute bacterial infection with either Gram+ or Gram- bacteria. This finding provided

novel insights into how the secretion of immune mediators can be influenced by an adaptor protein of the 14-3-3 family, during an acute bacterial infection.

Now that some of the major players involved in the route of AMP trafficking and the mechanism for vesicle exocytosis and cargo release have been identified in Drosophila (e.g., Rab4, Rab11, Syntaxin1A and 14-3-3), other important questions arise. What protein complexes are 14-3-3 proteins involved in to con-trol the delivery of exocytic vesicles to the cell surface, and how selective are the events that 14-3-3 regulates, during vesicle fusion at the plasma membrane? The phenotypic similarity between the silencing of 14-3-3 and the membrane fusion protein Syntaxin1A, suggests that this adaptor protein might have an impor-tant role in executing membrane fusion. Recent proteomic analysis[14] predicted a number of key Drosophila vesicular traf-ficking components that are potentially associated with 14-3-3. These proteins included Rab11, as well as the proteins of the exocyst complex, Sec5, Sec8, Sec10 and Sec15. The latter tetrameric exocyst complex serves to direct vesicles along microtubules from the Trans-Golgi Network (TGN), via recycling endo-somes, to the plasma membrane.[15] In the single celled eukaryotic bacterivores, Dictyostelium; 14-3-3 contributes to microtubule dynamics during cytokinesis, with the loss of 14-3-3 inhibiting this process.[16] There could have been a deregu-lation of the microtubule-related transport system in Drosophila *14-3-3* mutants, but the Rab11 vesicles observed in close vicinity to the plasma membrane of fat body cells, tended to exclude this possibil-ity. Another point of a 14-3-3 regulatory role might be cortical cytoskeleton, includ-ing actomyosin complex, known to be essential at the final stages of exocytosis at the cell periphery.[17] Small GTPases of Rho family, through the continuous reorganization of actin cytoskeleton, induce the movement of exocytic vesicles in cooperation with actin-binding pro-teins and the intracellular motor myosin. In doing so, Rho proteins facilitate vesi-cular delivery to the plasma membrane, membrane fusion and subsequently exocytosis.[18] Dictyostelium 14-3-3 is

necessary for the dynamic events, connect-ing microtubules to filamentous actin via controlling the Rac small GTPase of the Rho family, and myosin II.[16] The tether-ing of exocytic vesicles to specific sites on the plasma membrane of yeast cells is also mediated by members of the Rho GTPase family; CDC42 and Rho3.[19-21] The findings of a functional role for yeast 14-3-3/Bmh2p in polarized vesicular transport and bulk exocytosis, which also depends on modulating the actin cytoske-leton,[22] suggests that Drosophila 14-3-3ε might mediate its affect on exocytosis by regulating these Rho small GTPases. The activation of Rho GTPases in controlling downstream effectors (e.g., the actin motor myosin) is mediated by Guanine Nucleotide Exchange Factors (GEFs), and previous studies have established a role for mammalian 14-3-3β as a negative regulator of Rho-GEF function.[23,24] We explored the possibility that Drosophila *14-3-3ε* exocytic defects are mediated by Rho-GEF Pebble.[25] To do so, we reduced by half the level of Pebble expression in *14-3-3ε* mutants. Provided that these two proteins are involved in the same genetic pathway, this double mutant background (*14-3-3−/−*, *pebble+/−*) was expected to generate a dominant negative modification of the *14-3-3ε* phenotype. However, the heterozygosity for a strong allele of *pbl³* did not significantly alter the accumula-tion Rab11-GFP-positive vesicles in *14-3-3ε* mutants (unpublished observations), eliminating the possibility of this func-tional interaction in controlling exocytosis in fat body cells. Thus, although previous data showed the affects of human 14-3-3 on the RhoGEF-related propagation of exocytosis,[23] our results did not support this role in Drosophila fat body cells, or at least with the involvement of this particular RhoGEF. There is a clear need to accurately characterize the final events in the process of exocytosis, both in Drosophila and in mammalian models, and to establish a mechanism for 14-3-3's regulatory control of this important process.

In summary, regulation of vesicular trafficking and exocytosis near and at the plasma membrane is complex and still a rather undefined process. Proteins that regulate this stage of exocytosis include

GTP-binding proteins of Rab and Rho families, the proteins of the exocyst complex, microtubule and actin cytoskeleton, and their accessory proteins and proteins of the SNARE family that mediate membrane fusion. Our recent study identified 14-3-3 as a regulator of exocytosis in Drosophila immune response tissues. However, several questions require further investigation, including the mechanistic role of 14-3-3 in exocytosis, its role in the assembly of protein complexes and the specific consequences of the loss of 14-3-3 proteins in mammalian immune response cells.

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