

## Regulating Secretory Lysosomes

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Received May 28, 2006; accepted June 9, 2006

**Secretory lysosomes are lysosomes which are capable of undergoing regulated secretion in response to external stimuli. Many cells of the immune system use secretory lysosomes to release proteins involved in their specialised effector mechanisms. Precisely how lysosomal secretion is regulated in each of these cell types is now the study of much research as these mechanisms control the ability of each of these cells to function. Studies on a number of human genetic diseases have identified some key proteins in controlling secretory lysosome release, and now many interacting partners have been identified. The different regulatory components seem to vary from one cell type to another, providing a multitude of ways for fine tuning the release of secretory lysosomes.**

**Key words:** cytotoxic T lymphocyte, Rab27a, Munc 13-4, secretory lysosome.

Lysosomes are membrane-bound organelles that represent the main degradative compartment within the vacuolar system of the eukaryotic cell. The endocytosed material is initially sorted in the early endosomes (1) where lipids and proteins are either recycled back to the plasma membrane or proceed to late endosomes and eventually to lysosomes to be degraded (2). Lysosomes represent the endpoint of the endocytic pathway (3, 4) in which more than 40 acid hydrolases are stored and perform the catabolism of the cells at an optimum pH in the range of 4.6–5.0 (5).

Some cell types contain a specialised lysosomal compartment that can secrete its contents into the extracellular environment in response to an external stimulus (6). These organelles have been commonly referred to as “secretory lysosomes” or “lysosomal-related organelles” (LROs) and include melanosomes, lytic granules, Major Histocompatibility Class (MHC) II compartments (MIIC), platelet dense granules, basophil granules and azurophil granules (7). The importance of lysosomes and LROs in the maintenance of a healthy environment and in effector function of specialised cells is underlined by a multitude of genetic diseases that affect the correct packing and functioning of these organelles.

Conventional and secretory lysosomes share many morphological and biochemical features. The membrane of these organelles contains a set of highly glycosylated proteins and additional proteins that mediate the transfer of amino acids and a proton pump for the maintenance of an acidic luminal pH (8, 9) for the functioning of the acid hydrolases stored in their lumen. There are more than 40 acid hydrolases that perform lysosomal degradation which has a fundamental role in many physiological processes. Lysosomes maintain the cellular environment in a number of different ways. Their primary role is to degrade proteins, but in addition they play a role in down-regulating surface receptors, releasing endocytosed

nutrients and also repair of the plasma membrane after damage. In addition secretory lysosomes play key roles in immune defence at both innate and adaptive levels by secreting bio-active proteins for cell death as well as inactivating pathogenic organisms and by processing antigens for presentation (10).

### Secretory lysosomes

A number of different cell types contain secretory lysosomes. These can be a distinct subset of organelles, as in neutrophils, or indistinguishable from the lysosomes as in cytotoxic T lymphocytes (CTL). Secretory lysosomes take on a wide variety of roles in different cell types according to the proteins that each cell type synthesises and stores in secretory lysosomes. CTL and natural killer cells recognise and kill tumourigenic and virus-infected cells by releasing perforin and granzymes from secretory lysosomes. Basophils and mast cells release histamine stored in their lysosomes upon recognition of IgE to initiate an inflammatory response. Platelets release clotting agents stored in their dense granules to mediate blood clotting. Osteoclasts can fuse their lysosomes with the plasma membrane and release hydrolases into the extracellular space in order to digest and remodel bone. Antigen presenting cells degrade and process endocytosed material in order to load antigen onto MHC class II molecules. Melanocytes synthesise and store melanins in melanosomes prior to their transfer to keratinocytes. The majority of these cells derive from the haematopoietic lineage, the exception being melanocytes that originate from the neural crest (11).

CTL and Natural Killer (NK) cells play key roles in the immune system, patrolling the body and recognising tumourigenic and virally-infected cells in order to kill them. The formation of an immunological synapse (12, 13) at the point of contact between effector and target cell allows the recognition of an antigen, presented on MHC class I, by the T cell receptors of CTL. Recognition triggers rapid polarization of the secretory apparatus of the CTL. First, the microtubule organising centre

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(MTOC) polarizes towards the immunological synapse followed by the lytic granules which are transported along the microtubules in a minus direction towards the MTOC before being secreted at the plasma membrane (14, 15), delivering the lethal kiss of death to the target cell.

Lytic granules contain several molecules that mediate target cell death. Key to this process is perforin, a pore forming protein that is able to self-polymerise creating pores in the plasma membrane. Granzymes are also released from lytic granules and pass through the perforin pores to trigger rapid apoptosis of the target. Mutations in perforin have been associated with the onset of Familial Haemophagocytic Lymphohistiocytosis (FHL) type 2 (OMIM #603553) accounting for approximately 30% of the total diagnosed cases. The pathology is caused by the patient's inability to down-regulate an immune response often triggered by a viral infection. Activated lymphocytes continue to circulate and to produce inflammatory cytokines, such as IFN $\gamma$ , TNF $\alpha$ , IL-6, IL-10 and M-CSF, that induce the infiltration of organs and CNS by macrophages and lymphocytes (16, 17) leading to rapid death unless a bone marrow transplant is performed. Insights into the regulation of secretory lysosome release have also been gained from studies of a number of other human genetic diseases in which release of the perforin-containing secretory lysosome from CTL is disrupted. This in turn has led to the identification of interacting proteins which fine tune lysosomal secretion (Table 1).

### Adaptor protein-3

The Adaptor proteins 1, 2, 3 and 4 (AP-1, AP-2, AP-3, and AP-4) are all involved in the sorting of proteins from the trans-Golgi network or the endosome to different compartments in the cell. While it is clear that AP-1 and AP-2 bind to clathrin and assist in the formation of vesicles, less is known about the actions of AP-3, and AP-4. AP-3 contains consensus clathrin binding motifs, although it is unlikely to be involved functionally in clathrin mediated processes (18).

Hermansky-Pudlak syndrome (HPS) is an autosomal recessive disease that presents with a range of symptoms including platelet defects and oculocutaneous albinism. The pearl and mocha mice models for HPS, and human patients with HPS2 have mutations in AP-3 resulting in loss of protein (19, 20).

Fibroblasts from HPS2 patients show an increase in the levels of the lysosomal specific proteins CD63, lamp-1 and lamp-2 at the plasma membrane (21). In CTL derived from HPS2 patients granzyme A, granzyme B (both sorted by

the mannose phosphate receptor) and perforin (sorting pathway not known) are all sorted correctly to the secretory granules whereas CD63 is mis-sorted to the plasma membrane. Strikingly, CTL from these HPS2 patients are not able to kill targets. EM studies show that the secretory granules remain at the periphery of the cell, and do not migrate along microtubules towards the target cell (20). This suggests that these cells suffer from a defect in polarisation of the secretory granules. One possible explanation for this is that loss of AP-3 results in the mis-sorting of motor proteins required for microtubule mediated movement.

### CHS or Lyst protein

Chediak Higashi syndrome is a lethal disorder characterised by recurrent bacterial infections as a result of impaired immune function, bleeding defects and partial albinism. Most deaths occur as a result of lymphocyte infiltration during the "accelerated phase" of the disease, with the number of deaths caused by infection significantly reduced thanks to antibiotics. This disease affects many mammals including human, and the beige mouse (CHS model). In hematopoietic and related cells giant granules replace the normal LROs. To date mutations in only one protein, CHS or "Lyst," appear to be responsible for this disease (22).

The CHS/Beige gene encodes the Lyst protein, a putative cytosolic protein of 3801 amino acids and approximately 430 kDa. A series of twenty HEAT, or ARM, repeats make up the amino-terminus. These domains are believed to function in membrane interactions, particularly vesicle transport. The very carboxy-terminus contains a number of WD-40 repeats, suggesting a role for Lyst in a number of potential protein-protein interactions. In between these two regions lies a BEACH domain. As of yet, the function of Lyst is unknown (22).

Several studies on CTL and NK cells suggest a role for Lyst in the late stages of secretory lysosome biogenesis. When grown in tissue culture, resting CTL clones are activated and follow an approximate 21 day cycle. CTL proliferate and the new cells synthesise new granules from day 1 until day 15 which can be followed as the culture grows synchronously. The last six days involve down-regulation of killing machinery as the CTL return to the resting state. At day 7 after stimulation, CTL from Wild Type (WT) and CHS donors both contain a number of small lytic granules, yet at day 12 WT CTL contain multiple small lysosomes but their CHS counterparts contain one or two giant lysosomes (4). It would appear that lytic granule biogenesis is unaffected in CHS CTL but the absence of a functional Lyst protein results in the

Table 1. Membrane traffic diseases and related proteins.

Membrane traffic diseases	Location	Gene product	OMIM reference
GrisCELLI syndrome type 1/Elejalde syndrome	15q21	myosin Va	#214450
GrisCELLI syndrome type 2	15q21	Rab27a	#607624
GrisCELLI syndrome type 3	2q37	melanophilin	#609227
Chediak-Higashi syndrome	1q42.1-q42.2	Lyst	#214500
Hermansky-Pudlak syndrome type 2	5q14.1	Adaptor protein 3	#608233
Familial Haemophagocytic Lymphohistiocytosis type 3	17q25.1	Munc13-4	#608898
Familial Haemophagocytic Lymphohistiocytosis type 4	6q24	Syntaxin 11	#605014

formation of a huge lytic granule, most likely reflecting a defect in membrane fusion or fission during biogenesis.

In both cases perforin and granzyme A are sorted to the granules and co-localise with the lysosomal marker Lamp 2. However, the cytolytic activity of CTL and NK cells is severely decreased in CHS patients (23, 24). This lack of killing appears to stem from an inability of the CHS CTL to secrete the contents of the giant granules towards a target.

Less is known about the effects of *Lyst* mutations in other cell types. Platelet function is altered, resulting in prolonged bleeding times. In melanocytes, melanosomes are larger than normal and cluster in the perinuclear region. It is not clear which stage of melanocyte function is impaired, be it melanosome biogenesis, localisation to the periphery or transfer to keratinocytes (22). Nonetheless the cellular defects suggest a similar role for *Lyst* in melanocytes and CTL.

### Rab proteins

Rab proteins are a family of small Ras-like GTP binding proteins that regulate and control intracellular membrane trafficking events such as vesicle formation, organelle motility and the docking of vesicles to specific membranes. More than 60 Rab proteins are expressed in human and mice. They are closely related and display a high level of structural conservation. GTP bound Rabs are active, binding to their respective effector proteins. A “switch” to the GDP form “turns off” binding providing a simple mechanism to control interactions (25, 26).

In order to attach to membranes, Rabs must be prenylated. This post-translational modification is very hydrophobic and occurs with the assistance of two proteins. Rab Escort Protein (REP) interacts with unmodified Rab, forming a complex which is bound by Rab geranylgeranyl transferase (RGGT). Two prenyl groups are then added sequentially by RGGT to the Rab at two C-terminal cysteine residues. REP is proposed to transfer the prenylated Rab to its destination membrane (27, 28).

RGGT is important for correct secretory granule function in CTL. The gunmetal mouse is a model for Hermansky-Pudlak syndrome, a disease characterised by partial albinism and bleeding disorders. The  $\alpha$  subunit of RGGT is mutated, resulting in a 75% decrease in protein level and activity, and therefore a reduction in the prenylation of Rabs (29). CTL derived from gunmetal mice show a significant reduction in target cell killing, although they exhibit near WT levels of hexosaminidase secretion. Confocal images show that the secretory granules remain distributed around the periphery of the cell, rather than polarising at the immunological synapse, a feature most likely contributing to the lack of target killing. It would appear that one or more Rab proteins are required for lysosome movement in CTL and/or the sorting of lytic agents to the secretory lysosome. The underprenylation of Rab27a, a protein required for lytic granules to reach the plasma membrane at the immunological synapse, is also likely to contribute to the phenotype (15).

Rab27a is widely expressed across a range of tissues and cells, with a role in many non-neuronal, regulated secretory events including exocytosis of secretory granules from PC12 and adrenal chromaffin cells, insulin granule exocytosis,

antigen induced histamine release from RBL-2H3 cells and melanosome transport in melanocytes (30–34).

Griscelli syndrome (GS) is a rare autosomal recessive disease. Patients suffering from GS 2, and the ashen mouse model, display defects in pigmentation of hair and skin, incorrect localisation of melanosomes in melanocytes, and develop Hemophagocytic syndrome which results from mutations in, and loss of Rab27a. CTL derived from GS2 patients and ashen mice show a drastically reduced ability to kill targets. While secretory granule biogenesis and localisation appears normal, the final step of granule exocytosis is impaired. Secretory granules polarise towards the point of contact with the target but are seen to be clustered just behind the plasma membrane rather than fuse with it. This strongly suggests a role for Rab27a in the docking of granules to the plasma membrane (15, 35, 36).

### Munc 13 proteins

The *unc-13* protein expressed in *C.elegans*, and the Munc-13 family of proteins, Munc 13-1, -2, -3 and -4, expressed in mammals are structurally and functionally related. *unc-13*, and Munc 13-1, -2 and -3 all contain one C1 domain, two C2 domains, and two conserved Munc Homology Domains (MHDs), MHD1 and MHD2. *unc-13* and Munc13-1 also contain an extra C2 domain at the N-terminus of the protein, while Munc13-4 consists of two C2 domains, and the MHD1 and MHD2. A wonderful array of experiments in *C.elegans* and mammalian cell systems indicate a role for these proteins in the priming of synaptic and secretory vesicles in a step just preceding fusion with the plasma membrane (37, 38). The expression of Munc13-4 is limited to cells of haematopoietic lineage, where it plays a crucial role in the exocytosis of secretory lysosomes (39).

Patients suffering from FHL3 all have mutations in Munc13-4 that result in loss of protein, or the disruption of important functional domains through deletion, frameshift or mis-sense mutations. CTL clones from patients all show a severely reduced ability to kill target cells (39, 40). Munc13-4-EGFP transfected into patient cells can restore killing back to WT levels. RFP tagged Munc13-4 colocalises with perforin in WT control cells, suggesting that Munc13-4 is normally present on lytic granules. Granzyme A secretion is abolished in FHL3 cells, while secretion of IFN $\gamma$  is normal. This suggests that Munc13-4 is involved in the secretion of preformed lytic granules but is not required for the secretion of cytokines that utilise the constitutive secretion pathway. Interestingly, patient CTL can polarise as normal and secretory granules are seen docked with the plasma membrane. However, the secretory granules do not fuse with the membrane and there is no delivery of lytic granule content towards the target (39). These results strongly suggest that Munc13-4 functions after a Rab27a mediated docking step, to prime the machinery required for lytic granule fusion.

### Rab27a effector proteins

Rab27a interacts with a number of different effector proteins, in a range of cell types. These include the Synaptotagmin-like protein (Slp) family, that contain Slp



isoforms 1-5, rabphilin, Slac2 (melanophilin), and Noc2. These proteins all contain an N-terminal Slp Homology Domain (SHD) comprised of two alpha-helical domains that sometimes sandwich a Zn-finger domain. The SHD domain binds to Rab27a and Rab27b in a GTP dependent manner. Slp 1-5 and rabphilin all contain two C2 domains towards the C-terminus of the protein (25).

Melanophilin and Slp2a are required for the movement of melanosomes to the plasma membrane of melanocytes. Melanophilin binds to Rab27a and Myosin Va, forming a bridge between melanosomes and actin. When Rab27a or Myosin Va are missing in GS patients (35), or Slac-2a is knocked down via siRNA, melanosomes accumulate inside melanocytes. If Slp2a is knocked down in melanocytes, then melanosomes are seen to migrate towards the periphery, but cannot attach to the plasma membrane (41). This suggests that Rab27a interacts first with Slac-2a, to direct melanosomes toward the periphery and then interacts with Slp2a to mediate attachment to the plasma membrane.

Rab27a and Slp1 are both expressed in LNCaP carcinoma cells, where Slp1 is involved in the regulation of androgen-dependent secretion of prostate-specific antigen and prostate-specific acid phosphatase (42). Slp4a interacts with both the GTP and GDP bound Rab27a, and the SNARE priming factor Munc 18-1. The interaction with Rab27a-GDP appears to inhibit the exocytosis of dense-core vesicles in PC12 cells (43).

One other Rab27a effector protein is Munc13-4. Rab27a-GST was used to pull down Munc13-4 from platelet cytosol (44) and spleen cytosol (45), and confirmed as a direct interaction *via* pull down between Rab27a-GST and His tagged Munc13-4 (44). This interaction is dependent on the presence of GTP. GDP bound Rab27a or the Rab27a T23N mutant which does not bind GTP do not bind Munc13-4 (44, 45). The mild GS mutant Rab27a W73G, binds Munc13-4 significantly less well than Rab27a WT. *In vitro* binding studies show that Rab27a can bind to protein corresponding to Munc13-4 aa1-540 and aa240-917, but not to aa543-917. These results suggest that Rab27a binds Munc13-4 in the region aa240-543, which does not include the C2 domains or MHDs (Fig. 1). However, the same authors show that the FHL3 Munc13-4  $\Delta$ 608-611 mutant does not bind to Rab27a. These deleted amino acids correspond to a region of the MHD1 (45). Limited proteolysis of WT and FHL3 Munc13-4 suggests that the  $\Delta$ 608-611 mutation brings about a change in

conformation which abrogates binding between Rab27a and Munc 13-4.

Neeft *et al.* show that Rab27a and Munc13-4 colocalise with CD63 and serotonin at the secretory granules of RBL-2H3 mast cells. Expression of a number of GFP tagged Munc 13-4 constructs suggests that the two MHDs are responsible for localisation of Munc 13-4 to secretory lysosomes and that this localisation does not require an interaction with Rab27a. Density gradient separation of isolated platelets clearly shows Rab27a separating in the serotonin rich fractions and plasma membrane fractions, while Munc13-4 separates in the plasma membrane fractions alone (44). In an experiment that mimics mild Griscelli syndrome by reducing the level of functional Rab27a, addition of unprenylated Rab27a to platelets inhibits dense core granule exocytosis. This effect can be overcome by the addition of excess Munc13-4 (44).

Rab27a and Munc13-4 have an essential role in CTL and other haematopoietic cells, yet some questions remain unanswered. What is the mechanism by which secretory lysosomes transfer from the microtubules to the plasma membrane? Do Rab27a and Munc13-4 require other proteins to function? Answering these questions is key to understanding how CTLs kill.

#### SNARE proteins: Mediators of membrane fusion

Extensive research into the components required for the fusion of biological membranes in eukaryotic cells has highlighted the importance of the soluble *N*-ethyl maleimide sensitive factor (NSF) attachment protein receptor (SNARE) superfamily. In many, if not all fusion events, SNARE proteins and their regulators are essential for the direct, controlled and very rapid fusion of phospholipid membranes (46).

There are over thirty SNARE proteins found in mammalian cells. These consist of the Syntaxin, the Synaptosome-associated protein (SNAP) (-23, -25 or -29 kDa), and the Vesicle associated membrane protein (Vamp, also called Synaptobrevin) protein families. SNARE mediated membrane fusion occurs when a vesicle containing membrane bound Vamp is docked with a plasma (or target) membrane expressing Syntaxin and SNAP on the cytoplasmic leaflet. Priming of the SNARE components permits the formation of a SNARE complex, consisting of a Vamp, a Syntaxin and SNAP-25. SNARE motifs from these proteins wrap together creating a highly stable coiled-coil. It is believed

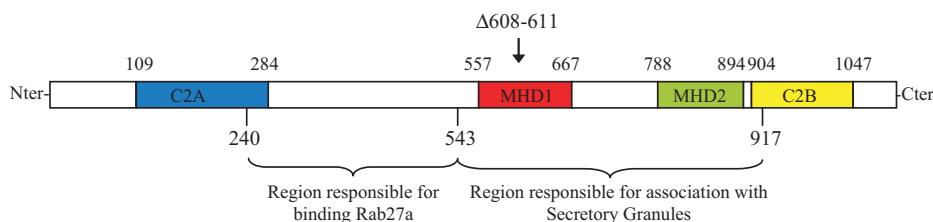


Fig. 1. **Munc 13-4.** This putative regulator of exocytosis is composed of at least four known domains. C2 domains towards the amino- and carboxy-termini enclose two Munc Homology Domains (MHDs). The MHDs are important for an association with secretory lysosomes and the region comprised of aa 240-543 is necessary for an

interaction with Rab27a (45). Presumably the C2 domains are important for binding the vesicle and/or the plasma membrane. An FHL3 mutation in MHD1,  $\Delta$ 608-611, abolishes localisation of the protein with secretory lysosomes.

that the energy released from the SNARE complex drives membrane fusion (46).

CTL mediated killing relies upon rapid, controlled and direct membrane fusion. The essential roles of Rab27a and Munc13-4 in the killing process demonstrate the regulated nature of this process, and strongly hint at the involvement of SNARE proteins. Extensive research shows that Rab mediated docking and Munc-13 priming precede SNARE mediated fusion.

So, what are the candidate SNAREs? SNAP-23 is found in the membrane fraction of lymphocytes and Jurkat cells (47), strongly suggesting a role in CTL. By analogy with neuronal systems, one would expect the need for a Vamp to be expressed on the granules and a syntaxin on the plasma membrane. Patients with FHL4 contain mutations in syntaxin 11 (47). However, expression of this protein has only been found in murine lymphocytes (48) and human monocytes, but is absent in human lymphocytes (47) suggesting that it is not a critical SNARE component involved in the exocytosis of secretory lysosomes from CTL. A number of other syntaxins have been reported in lymphoid cells and may play a role in secretory lysosome exocytosis. Interestingly syntaxin 6 has been shown to colocalise with the TGN, but in human neutrophils syntaxin 6 localises at the plasma membrane. Upon activation SNAP-23 bearing granules move to the plasma membrane, where syntaxin 6 and SNAP-23 are seen to co-localise (49). The combinations and localisations of SNARE components vary from one cell type to another and in phagocytes, Vamp3 and Syntaxin 4 are seen to be focused at the delivery site of phagosomal membranes to the plasma membrane (50). Precisely which SNAREs control the final fusion steps in each of the immune cell types using secretory lysosomes remains to be determined, but adds yet another layer of fine tuning to the regulation of lysosomal secretion.

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