Munc13-4 rab27 complex is specifically required for tethering secretory lysosomes at the plasma membrane

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Abstract

Cytotoxic T lymphocytes (CTLs) kill target cells through polarized release of lytic molecules from secretory lysosomes. Loss of munc13-4 function inhibits this process and causes Familial Hemophagocytic Lymphohistiocytosis type 3 (FHL3). Munc13-4 binds rab27a, but the necessity of the complex remains enigmatic, since studies in knock out models suggest separate functions. We here describe a non-canonical rab27a binding motif in the N-terminus of munc13-4. Point mutants in this sequence have severely impaired rab27a binding, allowing dissection of rab27a requirements in munc13-4 function. The munc13-4-rab27a complex is not needed for secretory lysosome maturation, as shown by complementation in CTLs from FHL3 patients, or in a mast cell line silenced for munc13-4. In contrast, fusion of secretory lysosomes with, and content release at the plasma membrane during degranulation, strictly required munc13-4-rab27a complex. Total Internal Reflection Fluorescence Microscopy Imaging reveals that the complex corrals motile secretory lysosomes beneath the plasma membrane during degranulation and controls their docking. The propensity to stall motility of secretory lysosomes is lost in cells expressing munc13-4 point mutants that don't bind rab27. In summary, these results uncovered a mechanism for tethering secretory lysosomes to the plasma membrane, that is essential for degranulation in immune cells.
**Introduction**

Natural Killer (NK) cells and cytotoxic T lymphocytes (CTL) are critical for immune responses against virus infections and cell transformation. The cytotoxic function is exerted through recognition of target cells, followed by regulated exocytosis of granzymes and perforin that causes apoptosis of the antigen presenting cell. The effector molecules are stored in lytic granules, otherwise known as secretory lysosomes or lysosome related organelles. Their molecular makeup comprises an acidic, proteoglycan core, soluble lysosomal enzymes and lysosome-associated membrane proteins. The efficient removal of target cells via the granule-dependent pathway also serves an important function in homeostasis of lymphocytes. Genetic defects affecting cytotoxicity cause persistent release of cytokines, including IFN-γ, IL-6, IL-18 and TNF, and uncontrolled expansion of CD8+ T lymphocytes and life-threatening macrophage activation syndrome.

After binding an antigen presenting cell, secretory lysosomes relocate along microtubules to the microtubule organizing center that polarizes towards the target cell. The centrosome and associated secretory lysosomes translocate together to the contact patch between effector and target cell, where docking of the centrosome delivers secretory lysosomes for fusion. This so called immunological synapse comprises a large, concentrically organized supramolecular complex of which cell adhesion molecules make up the peripheral zone while signaling proteins localize to the central core, where secretory lysosomes dock and fuse. Munc13-4 and Rab27a are essential for regulated release of secretory lysosomes in CTLs and NK cells. Genetic deficiency in Munc13-4 and Rab27a underlie the uncontrolled lymphocyte proliferation and activation observed in familial hemophagocytic lymphohistiocytosis (FHL) type 3 and Griscelli syndrome type 2 (GS2), respectively.

Rab27a is thought to be important for directing secretory lysosomes to the immunological synapse and perhaps for their release from microtubules. In munc13-4 deficient cytotoxic lymphocytes, secretory lysosomes appear to dock on, but do not fuse with the plasma membrane. Munc13-4 also regulates assembly of a exocytic precursor organelle from rab11 and rab7/rab27 containing endosomes, that is needed for the maturation of secretory lysosomes into a fusion competent lytic organelle. Rab GTPases are bimolecular switches whose active conformational states regulate membrane organization, protein sorting, and signaling through recruitment of a variety of often tissue specific effector proteins. Multiple effectors have been identified for rab27 of which three, including munc13-4 are expressed in...
cytotoxic lymphocytes \(^{14,15}\). Through the timed association with distinct effectors such as munc13-4, slp1, slp2-a and perhaps a myosin, rab27a can regulate the outcome of multiple cellular processes. Ultimately the coordinating function of rab27a positions secretory lysosomes at the immunological synapse and causes their fusion with the plasma membrane.

The molecular principles and signals underlying the directed membrane trafficking during secretory lysosome degranulation are not clear, and controversial with respect to the functional relationship between rab27s and munc13-4. Knockouts of rab27a/b and munc13-4 produce different secretion phenotypes in platelets \(^{16,17}\), while in neutrophils munc13-4 regulates motility of rab27 granules \(^{18}\). In CTLs and NK cells, rab27a and munc13-4 do not localize to lytic granules unless the cells are 'activated'. Separate signaling routes are used in NK cells to direct munc13-4 and rab27 to lytic granules \(^{19,20}\), while munc13-4 and rab27a associate independently with membrane\(^{10,21}\). We here identified a noncanonical rab27 binding region in munc13-4 that is required and sufficient for rab27a binding. Critical point mutants in this motif revealed an essential function of munc13-4 rab27a complex in tethering secretory lysosomes to the plasma membrane and in degranulation.
Materials and methods

Constructs

cDNAs encoding human rab11, rab27a, munc13-4, pMD2.G, and psPAX2 have been described \(^{22,23}\). Expression constructs and ala mutants of munc13-4 were generated by PCR and subcloned in pcDNA3.1HisB and pEGFP-C2. Following cDNAs were generously provided by indicated colleagues; pEF-BOS-Flag-rab27a (Mitsunori Fukuda, Fukuda Initiative Research Unit, RIKEN, Japan), Munc13-1 pcDNA3 (Nils Brose, MPI for Experimental Medicine, Göttingen), baiap3pcDNA3.1His (Takash Tokino, Institute of Medical Science Tokyo). cDNAs encoding fluorescent fusion proteins were extended with Gateway sequences and transferred to the lentiviral plasmid pLNT-SFFV-WPRE-Gateway \(^{23}\). All synthetic DNA was verified by dye termination sequencing of both strands.

Antibodies

The rabbit antibody against GFP was from BD Biosciences (Franklin Lakes, NJ). Mouse monoclonal antibodies were from the indicated sources; EEA1 and rat CD63 (BD Biosciences, San Diego, CA), GFP (Roche) and Flag M2 (Sigma), rat p80 (Juan Bonifacino, NIH). Rabbit antibodies against rab27a, and munc13-4 have been described \(^{21}\). Mouse monoclonal IgE antibodies against DNP (clone SPE-7) were obtained from Sigma. Conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cells, transfection, and lentiviral transduction

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood sample taken from controls (WT) and from a patient with Familial Hemophagocytic lymphohistiocytosis type 3 (FHL3) previously reported (P 83b in ref. \(^{5}\)). This patient carries a homozygous one base deletion (214delC) leading to frameshift and premature stop codon. Samples were obtained from patients and controls who had provided written informed consent in accordance with procedures at Institut National de la Santé et de la Recherche Médicale. CTLs were obtained as previously described \(^{10}\), and (co-) transfected using Amaxa nucleofection. Electroporated cell populations were incubated in complete medium for four hours and IL2 was then added. For each individual PBMC population electroporated, the “transfected” cell population designates the cell fraction that co-expresses the vectors whereas the “non” transfected cell fraction designates cells that do not express the vectors. Efficacy of transfection was around 30%. The degranulation assay was performed 6-8 hours after transfection. Lentivirus production in HEK293T cells and transduction of the RBL-2H3
mast cell line were described before. The following siRNAs (Applied Biosystems) GGAACAAGAUUUUUCACAAAtt (siRNA#1), and GUUGAAUGGUUUCACCUGAtt (siRNA#2) were used at 200 pmol to silence munc13-4 in RBL-2H3 cells.

Degranulation assays
To quantify cytotoxic granule exocytosis, CTLs were stimulated with 10 μg/ml of coated anti-CD3 mAbs in presence of anti-CD107a-FITC (fluorescein isothiocyanate) and anti-CD107b-FITC mAbs, according to a standard technique. Cells were incubated for 3 hours at 37°C in 5% CO2. Thereafter, cells were harvested, washed once with PBS, 0.02% azide, stained with anti-CD3-APC and anti-CD8-PE (BD Biosciences) and analyzed by flow cytometry. Cytotoxic granule exocytosis was measured by the induction of CD107 surface expression on CTLs (Δ CD107), calculated as the percentage of CD107+ CTLs stimulated with anti-CD3 and subtracted from the percentage of CD107+ of unstimulated CTLs. Dot plots were gated on CD3+CD8+ T cells and gates were set individually on the basis of T cells incubated with medium alone. Data were analyzed with FlowJo 8.8.4 software (TreeStar). Degranulation in RBL-2H3 cells was induced with 100 nM phorbol 12-myristate 13-acetate (PMA)/1 μM ionomycin or 50 ng/ml IgE - 500 ng/ml HSA-DNP and released β-hexosaminidase was measured fluorometrically. Both methods yielded similar β-hexosaminidase secretion efficiencies.

Protein interaction assays
In vitro pulldown experiments were done with GTPγS loaded GST-rab27a produced in E. coli BL21(DE3) and 35S-labeled proteins generated by in vitro-transcription translation as described. Bound proteins were resolved on 10 or 12.5 % SDS-PAA gels, visualized by phosphorimaging, and quantitated with ImageQuant software. Normalization was done with respect to the input signal for each mutant. For in vivo binding studies we transfected COS-7 cells with pEF-BOS-Flag-rab27a with or without peGFP-munc13-4(240-290), or peGFP-munc13-4(240-340). After 24 h, the cells were lysed and immunoprecipitations with Flag-M2 beads (Sigma) and Western blot with GFP antibodies was performed as described.

Fluorescence microscopy
Fluorescence microscopy on fixed specimen was performed as before. Coverslips were examined with a Zeiss LSM 510 confocal microscope (Oberkochen, Germany) using a 63x oil objective or on a Delta vision light microscope equipped with a Xenon lamp, a 100x oil
objective and collected on a chilled emCCD camera (Cascade, Photometrics). Detectors were set to detect an optimal signal below the saturation limits. Image sets to be compared were acquired during the same session and using the same acquisition settings. Images were deconvoluted using Softworx software (3.7.13EL); ratio aggressive, 15 iterations.

**Total Internal Relection Fluorescence (TIRF) Microscopy**

For TIRF experiments we used a objective type TIR fluorescence system based on an inverted microscope (Eclipse TE2000-U, Nikon) equipped with an oil-immersion objective (plan apo 60X NA 1.49, Nikon). Specimens were illuminated with a model 161C-030 argon laser (Spectraphysics), with an excitation power between 10-30 μW. Excitation light was separated with a dichroic mirror (Nikon B-2A) from fluorescent light originated from the sample and photons were collected on a chilled emCCD camera (Cascade, Photometrics). Winview software was used to acquire, display and process the images. Images were recorded (EM gain 3000) with a time lapse interval of 0.1 -1 sec. To generate the evanescent wave the laser was put at an angle of 65°, and the calculated evanescent wave field depth was 100 nm. Under these conditions one pixel correlated to 153 nm. Transfected RBL-2H3 were kept in 750 µl DMEM 1% FCS in a climate-controlled chamber with 5% CO2 at 37°C. Cells were activated by adding 250 µl medium containing PMA and ionomycin as described above.

**Quantitative image analysis**

Image analysis was performed with the Volocity software suite (version 5.2 Improvision). For colocalization of munc13-4 positive structures with endosomal markers we obtained z-stacks of at least 10 slices with optimal thickness for the pinhole. On the Delta vision we images slices of 0.15 μm. We defined structures with Volocity in each channel by % intensity, and separated the structures by size (< 0.5 μm). Thresholds for intensity were manually set on control samples and 3D volumes were obtained. For analysis of size distribution, the average of 3D volumes were used. Positioning and relative position to the nucleus were evaluated manually on a single slice. To find overlap between structures identified in two independent channels, the 3D volumes were analysed for overlap. For colocalization statistics the total overlapping volume was compared to the total amount of volume per channel. Two-dimensional tracking of vesicles was done using the particle tracking plugin of imageJ using 0.3 μm for detection, displacement 4, link range 2. This yielded the highest amount of tracks without non-sense jumps or fragmentation. On average...
60 tracks were analyzed per cell for 15 cells in 3 experiments, tracks shorter than 10 frames were discarded. Track coordinates were analyzed using Excel, Means Square Displacement (MSD) was determined by plotting the distance between the first and last coordinate and plotted against Δ time. Granules were defined immobile if the displacement at the last track was not larger than the detection limit of the granule (0.3 µm).

Statistical analysis

Statistical analysis of data was performed on the average of three independent experiments. To evaluate the statistical significance we used single factor ANOVA and t-tests for individual significance between two samples. p values below 0.05 were considered statistically significant. * p < 0.05, ** p < 0.01, and ***p < 0.005.
Results

**Munc13-4 contains a novel rab27 binding motif**

The molecular principles underlying directed membrane trafficking during degranulation of immune cells are not clear. Munc13-4 is an unusual rab27 effector in this pathway because it lacks a conserved rab27 binding domain (R27BD), does not need rab27 interaction for membrane association and is the only known essential rab27 effector in cytotoxic lymphocytes. Rab27 binding requires aa 240-543, but more precise information is not available. Interaction assays with additional truncations in GST pull downs showed that aa 240-543 is sufficient for rab27 binding (Fig. 1A,B). N-terminal shortening of this munc13-4 fragment to 290-543 removed most of the binding capacity, suggesting that a major rab27 binding region is within amino acid 240-290 (Fig. 1B), which contains the last residues of the first C2 domain, also known as C2A domain. Complementary coIP experiments of FLAG-rab27 with GFP-munc13-4(240-290) confirmed that this short construct retained the ability to bind rab27 in vivo (Fig. 1C). We constructed a model for the 3D structure of munc13-4 C2A on the basis of the equivalent munc13-1 C2B domain structure (Fig. 1E, see figure legend for details). Amino acids 240-290 encompass the C-terminal part (aa 240-284) of the C2A domain (aa 109-284), including its three last β-strands (designated here βA, βB and βC). This region is conserved among munc13 proteins but the rab27 binding determinant is specific for munc13-4 (Fig. S1A). In accord with this, we found little if any binding to other munc13 family members (Fig. S1B). We then performed alanine scan mutagenesis on aa 240-291 in full length munc13-4, by replacing sequential groups of 3 amino acids with alanine residues. Interaction assays with the point mutants, showed that munc13-4(FQL>AAA) and munc13-4(IHK>AAA) mutants lost 80% rab27a binding, while a gapped construct munc13-4Δ(280-285) lacking the FQLIHK residues, confirmed their requirement (Fig. 1F). The sequence of this region is only modestly conserved among munc13 family members (Fig. S1A). Flanking amino acids contributed to the interaction surface since mutations in these gave an intermediate binding phenotype (Fig. 1D). YFP-munc13-4(FQL>AAA) and YFP-munc13-4Δ(280-285) localized correctly with CD63+ lysosomes in RBL-2H3 cells (Fig. S2, left column), and quantitation of secretory lysosome diameter, localization and distance to the nucleus all showed that these were not significantly affected (not shown). A small fraction (<10%) of wild type munc13-4 and the mutants localized to early endosomes containing EEA1 (Fig. S2, right column). Because misfolded munc13-4 is distributed entirely to the cytoplasm, and the two mutants are associated with CD63 structures (Fig. S2, right column), impaired rab27a binding is not an indirect effect caused by misfolding. Limited
proteolysis experiments of the mutants with endoproteinase Glu-C and proteinase K gave the same digestion patterns as wild type protein by SDS-PAGE (not shown), whereas the munc13-4Δ(608-611) FHL3 mutant does not 21. Taken together, these experiments show that rab27a binding is not required for localization of munc13-4 to secretory lysosomes or for secretory lysosome positioning.

*Munc13-4-rab27 interaction is required for CTL degranulation*

We next analyzed functionality of the munc13-4 - rab27a complex in secretory lysosome (cytotoxic granule) exocytosis. CFP-tagged munc13-4 constructs were expressed in munc13-4 deficient cytotoxic T cells (CTLs) from an FHL3 patient, who represents a natural human munc13-4 knockout model. Degranulation was then evaluated by the appearance of the granule-associated CD107 on the surface of CTLs following cell stimulation with an antibody against the T cell receptor CD3 subunit. Like the FHL3 munc13-4Δ(608-611) mutant and in contrast to wild type munc13-4, point mutations in R27BD, as well as munc13-4Δ(280-285) were unable to rescue degranulation (Fig. 2A,B). Mutant construct munc13-4(PDR>AAA) which preserved rab27a binding, restored degranulation to similar levels as wild-type munc13-4, while munc13-4(RRA>AAA) with intermediate binding gave a partial rescue (Fig. 2A,B). Thus the molecular interaction between munc13-4 and rab27a regulates a critical step of the cytotoxic granule pathway in lymphocytes.

*Munc13-4 interaction with rab27 is essential for degranulation in RBL-2H3 cells*

We next investigated if this role of munc13-4 and rab27a was specific for CTLs or might occur as well in immune cells that degranulate in a nonpolarized manner 21,27,28 via compound exocytosis. This exocytosis modality allows for the instantaneous release of secretory lysosome content 29 upon immune receptor signaling. We used a robust complementation assay in RBL-2H3 cells 23 in which siRNA resistant YFP-munc13-4 constructs are expressed from lentiviruses, and endogenous rat munc13-4 is knocked down to < 15% by two siRNAs (Fig. 2C). FcεR was crosslinked with IgE and TNP-BSA and stimulated release of β-hexosaminidase was used as read-out for secretory lysosome degranulation. In accord with degranulation experiments in FHL3 cytotoxic lymphocytes, munc13-4 mutants that poorly bind rab27a, also yielded minimal recovery in mast cell degranulation (Fig. 2D). Degranulation was less efficiently inhibited in mast cells than in FHL3 CTLs, which probably reflects the small residual level of non-silenced munc13-4.
Taken together we found that munc13-4 rab27 complex is essential in secretory lysosome release in immune cells.

**Munc13-4 regulates merger of rab11 and rab27a endosomes, independent of rab27a**

Munc13-4 serves a role in maturation of secretory lysosomes where it is needed for merger of rab27a containing late endosomes and rab11-positive recycling endosomes into a precursor exocytic endosome. Using the munc13-4 R27BD point mutants we determined whether this function of munc13-4 involves rab27a. In FHL3 CTLs cotransfected with DsRed-rab11, GFP-rab27a and CFP-munc13-4 constructs, we analyzed the co-distribution of rab11- and rab27a-labeled structures (Fig. 3A). Ectopically expressed munc13-4 or munc13-4 R27BD point mutants, similarly increased the number of merged rab11+ and rab27a+ structures compared to control CTLs or those from a FHL3 patient. We then investigated if the requirement for munc13-4 in maturation of secretory lysosomes could be extended to other immune cells. Introduction of YFP-munc13-4 in RBL-2H3 cells expressing CFP-rab11 and Cherry-rab27a, gave a significant increase in colocalization between rab11 and rab27a, compared to cells that were not transfected with YFP-munc13-4 (Fig. S3A and B), irrespective of their activation state. Knock-down of endogenous munc13-4 did not significantly affect the extent of colocalization between CFP-rab11 and Cherry-rab27a compared to nonsilenced control cells (Fig. S3). Possibly residual endogenous munc13-4 (Fig. 2) was sufficient to sustain coalescence of rab11 and rab27a membranes (Fig. S3B). When we combined silencing of endogenous munc13-4 with expression of YFP-munc13-4A(280-285), we obtained nearly identical colocalization for rab11 and rab27a as with wild type YFP-munc13-4, in resting (Fig. 3B-C) and activated (not shown) cells. Collectively the localization experiments confirmed a function for munc13-4 in maturation of secretory lysosomes. These observations extend previous studies in CTLs from GS2 patients where munc13-4 increased coupling of rab11 and late endocytic organelles in the absence of rab27a.

**Impaired rab27 binding reduces tethering of munc13-4 vesicles**

To understand how rab27 and munc13-4 coordinately control secretory lysosome release, we analyzed their dynamic behaviour in RBL-2H3 cells expressing Cherry-rab27a and YFP-munc13-4 using total internal reflection fluorescence microscopy (TIRFM). We saw two populations of YFP-munc13-4 - Cherry-rab27a containing granules (Fig. 4A). A class of small 0.3 µm subplasmalemomal structures that was distinct in diameter from the stationary
large (> 2 µm) multi-granular clusters observed by epifluorescence microscopy in the perinuclear cytoplasm (Fig. S2, left column). The smaller peripheral granules were mobile and moved from the interior of the cell to the TIRF zone and back. The large clusters seldom appeared in the TIRF zone (Fig. 4B) and were less fluorescent than the small granules (Fig. 4A).

Cherry-rab27a YFP-munc13-4 cells were then silenced for endogenous munc13-4 and we quantified the mobility of Cherry-rab27a YFP-munc13-4 granules by assaying their trajectories using TIRFM. The mean square displacement (MSD) of the trajectories was plotted against time interval (Δt) averaged over all tracks. We also scored the fraction of mobile granules, defined as granules with MSD larger than their own diameter of 0.3 µm. In Cherry-rab27a YFP-munc13-4 cells, many (~ 50%) of the granules were immobile at the plasma membrane while others made diffuse tracks along the cell surface. After activation by ionomycin and PMA, the granules became less mobile (movies 1, 2) which resulted in diminished displacement (Fig. 4A, C). Importantly, the fraction of mobile granules was significantly decreased in the activated cells, suggesting increased entrapment and coralling of the granules at the plasma membrane (Fig. 4C, E) in parallel with the stimulated release of β-hexosaminidase (Fig. 2D). MSD vs time interval (Δt) plots show negative curvature and will therefore eventually approach an asymptote, reflecting space-constrained movement. The radius of the constrained space is defined by the square root of the asymptote value. For Cherry-rab27a YFP-munc13-4 granules, the radius was reduced from 0.57 µm to 0.41 µm upon activation. Impaired mobility in this analysis (Fig. 4C, E) reflects enhanced tethering of the granules to the plasma membrane, as was originally shown for dense core granules in neuroendocrine cells 30-32.

In cells expressing YFP-munc13-4(FQL>AAA) and YFP-munc13-4Δ(280-285) mutants, the typical activation-induced mobility changes were not observed (Fig. 4D, E). This suggested that the rab27 binding mutants have reduced tethering/docking capabilities and that the interaction of munc13-4 with rab27a is needed to allow granules to effectively dock and fuse at the plasma membrane. Although Cherry and YFP fluorophores could not be simultaneously imaged on our microscopy system, we consistently obtained identical data for Cherry-rab27a and YFP-tagged constructs in the double transduced cell line. To rule out that the activity dependent change in mobility is caused by overexpression of YFP-munc13-4, we used Cherry-rab27a single transfectants and obtained the same results (data not shown).
Collectively the TIRFM experiments showed that the mutants have reduced tethering-docking capabilities and that the interaction of munc13-4 with rab27a is needed to allow granules to effectively dock at the plasma membrane.

**Discussion**

Cytotoxic lymphocytes harbor distinct secretory compartments for storage of cytokines and lytic molecules whose release occurs via different signaling and trafficking pathways. Conceivably the orchestration of these events requires sophisticated levels of control. Here we describe a general mechanism for regulation of secretory lysosome release by a complex of the small GTPase rab27 and munc13-4. First, we defined a new rab27a binding motif on munc13-4, which allowed the dissection of rab27-dependent and independent functions. Second, we showed that point mutants with impaired rab27a binding fail to complement loss of degranulation because tethering secretory lysosomes to the plasma membrane is inhibited. Third, we found that the rab27-independent function of munc13-4 is a general property of hematopoietic cells for secretory lysosome maturation.

The 3D structures of rab27b with melanophilin and rab27a with Slp-2a define a conserved R27BD, formed by two linked α-helical regions. Munc13-4 aa 240-290 does not resemble this binding site. It includes the last three β-strands of the C2A domain (aa 240-284, Fig. 1E) and a basic sequence (aa 285-290), which links the C2A domain to the following MUN domain. The critical amino acids 280-285, are at the edge of C2A and therefore outside the context of the full-length C2A domain. The structure of the 240-284 segment as present in full-length globular and well-folded C2A domain may be lost in the truncations studied here. On the other hand, because in the complete C2A domain, strand βB forms a β-hairpin with the βC-strand, which includes the critical 280-285 residues, it is attractive to suggest that such a local structure is maintained in the truncated proteins. Moreover, the partners of the amino acid residues which in strand βC participate in the hydrophobic core of the C2A domain (F280, L282), are found in strand βA (L248) and in the loop linking βA and βB (L253). Therefore, it is a distinct possibility that a local “C2A-like” structure is maintained in the truncated proteins, which permits binding of rab27a at the level of the bottom of the C2A domain and of its C-terminal extension (Fig. 2B). This hypothesis is supported by the fact that a similar region of the C2 domain (bottom tip and its C-terminal extension) is involved in the munc13-1 C2A domain heterodimerization with the RIM Zn-finger. Accordingly, the substitutions of aa 285-290 in alanine residues should either disrupt the fold (F280, L282)
or interfere directly in the binding event (Q281, I283, H284, K285). The correct localization of the munc13-4 constructs suggests that the second hypothesis should be preferred.

Elucidation of the rab27a binding region was essential for understanding the role of the multidomain protein munc13-4 in secretory lysosome functions. Particularly since the significance of the interaction with rab27 has been enigmatic. In resting mast cells, they colocalize extensively on the limiting membrane of secretory lysosomes \(^{21}\), suggesting constitutive residence of the complex at this location. In NK cells however, an external signal is required for colocalization of rab27 and munc13-4 \(^{19}\). In CTLs, munc13-4 codistributes with the recycling endosome marker rab11 and is important for their merging with rab27\(^{+}\) late endocytic organelles. Our functional localization experiments of rab11 and rab27 in CTLs and RBL-2H3 showed that munc13-4, irrespective of rab27 binding directs the merger of exocytic rab11\(^{+}\) and late endosomal rab27\(^{+}\) compartments. It is also in accord with the absence of a maturation phenotype in CTLs of GS2 patients \(^{10}\). It will be interesting to understand how munc13-4 can bring together rab11 and rab27 membranes. Possible mechanisms include scenarios where munc13-4 in complex with an accessory protein binds directly to rab11. Alternatively it could sample the rab11 effector network to bridge rab27 and rab11 membranes. The formation of secretory lysosomes, and the connections between them and with other granules have only recently become subject of investigation. The propensity to use munc13-4 for bringing together rab11 and rab27 compartments, suggests a fundamental role of rab11 containing recycling endosomes in supplying exocytic machinery \(^{37,38}\) to immature secretory lysosomes in immune cells. KIF13A and the adaptor AP-1 are two other regulators of traffic through recycling endosomes that have been implicated in function of melanosomes, a secretory lysosome related organelle \(^{39}\). This complex establishes a recycling endosomal domain that partially overlaps with rab11 \(^{39}\) and might cooperate in cargo sorting because it positions endosomes close to secretory lysosomes in the cell periphery, facilitating interorganellar connections. The aggregate data provide strong arguments for a role of recycling endosomes with rab11 in maturation of secretory lysosomes. Although this is an appealing general mechanism, cell-type specific regulation needs to be imposed since melanocytes for instance do not express munc13-4 \(^{21}\). This might however merely reflect the deployment of alternative rab11 effector networks in different cell types for related purposes.
What could be the molecular mechanism for the activity of rab27 munc13-4 complex? Other munc13's share the canonical organization of tandem C2 domains separated by the MUN domain 26. In contrast to munc13-4, however, they do not bind rab27. Instead the N-terminal extension of munc13-1, serves as an interaction node with rab3 and matrix proteins, that regulates the activity of presynaptic neurons 40. In munc13-4 this property might be conferred by interactions at the interface of the C2A and MUN domains with rab27. The MUN domain of munc13-1 can bind syntaxin-1 and SNAP25 in liposomes, and competes with munc18-1 41 to promote SNARE complex assembly. The homologous region in munc13-4 could similarly regulate syntaxin-11 42 and SNAP 23 43, thought to be involved in late stages of lysosome secretion. The v-SNARE VAMP8 is also required for exocytosis of lytic granules 44, and binds SNAP23 45, suggesting a functional relation between syntaxin-11, SNAP23 and VAMP8 in secretory lysosome fusion with the plasma membrane. The interaction with rab27a-GTP and Doc2α 46 within the MUN domain, conceivably controls the activity of the MUN domain in removing munc18-2 and opening syntaxin-11 (Fig. 5). Since docking and priming steps in membrane fusion are dependent processes 47, it is likely that the role of rab27a and munc13-4 might extend beyond priming. The MUN domain shares homology with helical rod components of the CATCHR family of tethering complexes, including sec6 of the exocyst, and vps53 of GARP 48,49. Possibly the complex of rab27 and munc13-4 forms a coincidence detection unit for recruitment of tethering factors such as slp family members to facilitate the capture and retention of secretory lysosomes at the plasma membrane (Fig. 5), and enhance the probability for assembly of cognate SNARE complexes.

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Authorship
The authors have made the following declarations about their contributions: Conceived and designed the experiments: EDE, MN, NTN, JV, PMPvBH, GdSB, PvdS. Performed the
experiments: MC, EDE, MG, MN, NTN, PvdS, JV. Analyzed the data: EDE, HCG, MN, NTN, JV, IC, PMPvBH, GdSB, PvdS. Wrote the paper: GdSB, PvdS. Conflict-of-interest disclosure: The authors declare no competing financial interests.

References


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Figures legends

**Figure 1: Critical residues in munc13-4 required for rab27 binding**

(A) Organization of munc13-4 and truncations used in this study. (B) Binding assay of $^{35}$S-labeled His$_6$munc13-4 truncations and GSTrab27aGTP$_{γS}$. Bound proteins were resolved on a 10% SDS-PAA gel and visualized by phosphorimaging. (C) FLAG-tagged rab27a and GFP-munc13-4 truncations were co-expressed in COS-7 cells. Anti FLAG immunoprecipitates were analyzed by Western blot with a monoclonal GFP antibody. (D) Binding assay of $^{35}$S-labeled His$_6$munc13-4 AAA mutants and GSTrab27aGTP$_{γS}$. Bound protein was eluted, and assayed by phosphorimaging. The amino acid sequence containing the R27BD is given at the bottom of the panel, and the coloring of the blocks indicates % binding as per the legend in the panel. (E) Model of the three-dimensional structure of the C2A domain of munc13-4 (aa 111-284, ribbon diagram). The model was made on the basis of the alignment of the C2A domain of human munc13-4 with the C2B domain of rat munc13-1, whose experimental 3D structure (pdb 3kwu) has been solved. The limits of the 240-284 segment, which includes the β-strands βA to βC, are shown, as well as the amino acids discussed in the text. Loops which were not modeled are symbolized with dashed lines. Amino acids of the Ca$^{2+}$-binding site are shown, together with the bound Ca$^{2+}$ ions (green spheres). (F) Binding assay of $^{35}$S-labeled His$_6$munc13-4Δ(280-285) or His$_6$munc13-4 and GSTrab27aGTP$_{γS}$. Bound protein was eluted, and assayed by phosphorimaging. Quantitations of signals present in bound and total samples, and background correction were done with the Imagequant software package (GE-Healthcare Life Sciences)

**Figure 2: Munc13-4 - rab27a interaction is required for degranulation**

(A) CTLs from control (WT-CTLs) or munc13-4-deficient patient (FHL3-CTLs) were transfected with CFP-munc13-4 wild-type (WT) or CFP-munc13-4 (FQL>AAA) mutant construct. In each experiment, the CFP-positive (transfected) and CFP-negative (nontransfected) cell populations were tested for CD107 expression after stimulation with anti-CD3. Numbers indicate the percentage of degranulating CTLs. The data shown are representative of 3 independent experiments with similar results. (B) CTLs from control (WT) or munc13-4-deficient patient (FHL3) were transfected with various CFP-munc13-4 constructs (WT or mutants) as indicated and the transfected (gray) and untransfected (black) cell populations were analyzed as in A. Values represent mean (± SD) percentages of the CD107+ CTLs. The results shown are representative of 3 independent experiments with similar results. (C) RBL-2H3 cells with and without ectopically expressed YFP-munc13-4
were transfected with two siRNAs targeting different regions in rat munc13-4 by AMAXA nucleofaction. Cell lysates were analyzed by Western blot with a polyclonal antibody against munc13-4 and a monoclonal antibody against actin. **(D)** RBL-2H3 cells stably expressing indicated wild type and mutant YFP-munc13-4 constructs were transfected with siRNA #2 or control siRNA as in A. Cells were activated as described in materials and methods and β-hexosaminidase activity was determined colorometrically. Percentage secreted is calculated as fraction of total amount of β-hexosaminidase activity present in a parallel dish of the same cell line that was not stimulated. We then set the extent of secretion in the cells treated with scrambled siRNA to 100%, and normalized the other results to this value. *** p < 0.005.

**Figure 3: Endosome coalescence occurs independently of munc13-4 rab27 complex**

**(A)** Quantitative analysis of rab11-rab27a overlapping structures in control or munc13-4 deficient (FHL3) CTLs co-expressing DsRed-rab11 and GFP-rab27a as previously reported. FHL3 CTLs were also co-transfected with wild-type (WT) or munc13-4(FQL>AAA) construct. Data represent statistical image analysis of two color pixel intensity correlation in all optical sections and are average (± SD) of overlapping surface calculated by computer-assisted image analysis of at least ten cells. **(B)** Confocal microscopy of RBL-2H3 cells stably expressing CFP-rab11 (blue), Cherry-rab27a (red) and YFP-munc13-4 constructs (green) while endogenous rat munc 13-4 was silenced with siRNA #2. **(C)** Quantitation of codistribution of rab11 with munc13-4 and rab27a. Colocalization was determined in 10 cells using Volocity according to material and methods and is displayed as a percentage of rab11 over munc13-4 (black) and rab11 over rab27a (gray). Error bars are SEM of three experiments. Scale bars are 5 µm. ** p < 0.01.

**Figure 4: Docking of munc13-4 granules at plasma membrane requires rab27**

**(A)** TIRFM view of YFP-munc13-4 in resting and activated RBL-2H3 stably expressing Cherry-rab27a and YFP-munc13-4. White trajectories represent tracks of individual granules. Scale bar denotes 2 µm. **(B)** RBL-2H3 cells expressing YFP-munc13-4 were imaged with a confocal microscope using 0.35 µm slice thickness. The smaller granules (closed arrowheads) that are observed in the TIRF view are predominantly found at the basal side of the cell (z=0 µm), while the signal for out of focus larger granules in the background is indicated with open arrowheads. Note that the optical sections are ~ 3 times thicker than the TIRF zone. The larger granules with diameter > 0.3 µm are distributed to the upper layers of the z-stack. Scale bar: 5 µm. **(C)** MSD vs. Δtime plots of the tracks analyzed per cell (n=15)
in resting (closed circles) or activated (closed squares) Cherry-rab27a YFP-munc13-4 expressing cells of a representative experiment. Black lines indicate the slope of the first 8 seconds (D) Same analysis as in C for Cherry-rab27a YFP-munc13-4(FQL>AAA) and Cherry-rab27a YFP-munc13-4Δ(280-285) cell lines. (E) Decrease in mobile vesicles after activation was analyzed per cell for 15 cells in 3 experiments and compared between wild type munc13-4 and mutants that do not bind rab27.

Figure 5: Hypothetical model for function of munc13-4 and rab27
Activation of cells targets munc13-4 and rab27 to each other's vicinity establishing a specific domain on secretory lysosomes. Colocalization facilitates the interaction between the two proteins and since rab27 and munc13-4 have independent membrane binding determinants, their interaction enhances the affinity of the complex for the secretory lysosome membrane. At this point the interaction with rab27 could fold munc13-4 in a conformation that allows the first C2 domain to bridge the apposing bilayers of secretory lysosome and plasma membrane. The complex can now serve as nucleation point or coincidence detection unit for interaction with other proteins important for secretory lysosome release. The C-terminal part of munc13-4 might bind to syntaxin-11 or engage Doc2α which then interacts with SNAP-23, establishing a regulatory circuit for controlling a cognate SNARE complex for fusion with the plasma membrane. The interaction with rab27-GTP secures the recruitment of slp2 and other effectors involved in tethering the secretory lysosome to the plasma membrane. Loss of rab27 binding uncouples these events and interferes with degranulation.
Figure 1

A

B

C

D

E

F
Figure 3

(A) Bar graph showing the percentage of rab11/rab27 colocalization. The x-axis represents different conditions: FHL3, control, FHL3 WT, and FHL3 FQL>AAA. The y-axis shows the percentage of colocalization.

(B) Fluorescence images showing the expression of CFP-munc13-4, YFP-munc13-4Δ(280-285), and Cherry-rab27a in different conditions. The images are merged to show colocalization.

(C) Bar graph showing the colocalization percentage of rab11/rab27a and rab11/munc13-4 under siRNA 2 conditions with YFP-munc13-4 WT and Δ(280-285).
Figure 4

Panel A: Comparison of resting and activated states for YFP-munc13-4. The images show a reduction in track density for activated vs. resting conditions.

Panel B: Confocal images at different z-planes (0, 0.35, 0.7, and 1.05 μm) illustrating YFP-munc13-4 expression.

Panel C: MSD (μm²) vs. Δt (s) graphs for wild type, showing distinct lines for resting and active states.

Panel D: MSD (μm²) vs. Δt (s) graphs for FQL>AAA and Δ(280-285), with similar differentiation for resting and active states.

Panel E: Bar graph comparing the reduction of tracks > 0.3 μm (%) across wild type, FQL>AAA, and Δ(280-285) conditions.
Figure 5
Munc13-4 rab27 complex is specifically required for tethering secretory lysosomes at the plasma membrane

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