CHAPTER THIRTEEN

Methods for Analysis of rab27a/munc13-4 in Secretory Lysosome Release in Hematopoietic Cells

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Contents

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Abstract

Secretory lysosomes constitute a heterogeneous organelle of hematopoietic cells that combines the properties of regular lysosomes with those of secretory granules. Although secretory lysosomes serve essential functions, such as in the immune system and blood clotting, the mechanisms underlying the release of contents are incompletely understood. It is clear, however, that rab27a and the C2 domain protein munc13-4 serve essential functions. Mutations in these genes lead to immune disorders where the lytic granule function of cytotoxic T cells is jeopardized in humans. We identified munc13-4 as a rab27a binding protein from spleen. Munc13-4 is highly expressed in several hematopoietic cells including cytotoxic T cells and mast cells. We describe the molecular features of the interaction and requirements for localization, and show that munc13-4 is a positive regulator of secretory lysosome exocytosis.

1. Introduction

Secretory lysosomes, also known as lysosome-related organelles, constitute a hybrid organelle that combines the functions of lysosomes and secretory granules (Stinchcombe *et al.*, 2004). In accordance with properties of lysosomes, they have an acidic lumenal pH, a complement of lysosomal enzymes, and lysosome-associated membrane glycoproteins. They often contain lumenal structures, and at the ultrastructural level appear to be related to multivesicular bodies (Peters *et al.*, 1991). Secretory lysosomes occur in cells of the hematopoietic lineage, and have been investigated particularly in cytotoxic T cells (CTLs), platelets, mast cells, dendritic cells, and B cells. Stimulation of immune receptors such as the T-cell receptor triggers signaling pathways that generate output via a variety of downstream effector systems. These include the degranulation of secretory lysosomes, which releases bioactive compounds like granzymes and perforin in CTLs, and of serotonin in mast cells (Gilfillan and Tkaczyk, 2006; Radoja *et al.*, 2006).

Melanosomes, the organelles that produce and store pigment in melanocytes, and retinal pigment epithelium are also considered secretory lysosomes (Marks *et al.*, 2003). In spite of the relationship between melanosomes and secretory lysosomes, they employ distinct machinery for docking and fusion with the cell surface. This notion is dramatically demonstrated by the case of human disease mutations in melanophilin (Griscelli syndrome type 3, GS3) and myosin-Va (Griscelli syndrome type 1, GS1) that preclude the formation of a ternary complex with rab27a, and thereby interfere with peripheral distribution of melanosomes and transfer of pigment

to keratinocytes. Patients with GS1 (Pastural *et al.*, 1997) and GS3 (Menasche *et al.*, 2003) have normal degranulation of secretory lysosomes in CTLs, while the lack of functional rab27a in Griscelli syndrome 2 (GS2) also prevents docking and degranulation of lytic granules (Menasche *et al.*, 2000), suggesting that secretion from lysosomes in CTLs requires other factors that cooperate with rab27a.

To find proteins involved in the release of secretory lysosomes, we performed preparative pull-down assays using rab27a-GTP and pig spleen cytosol. The rationale for this approach is that rab27a is highly expressed in the spleen (Seabra *et al.*, 1995), that spleen is enriched in hematopoietic cells, and that fresh spleens can easily be obtained from a slaughterhouse. Using this approach, we found munc13-4 (Neeft *et al.*, 2005), a distant relative of the MHD and C2 domain–containing munc13 proteins, that with the exception of a ubiquitously expressed munc13-2 splice variant, are predominantly expressed in the nervous system (Augustin *et al.*, 1999; Betz *et al.*, 1998; Koch *et al.*, 2000). Munc13-1 serves as a priming factor of glutamatergic synaptic vesicles (Rosenmund *et al.*, 2002), and perhaps as well for fusion of insulin granules in the β cells of the pancreas (Kwan *et al.*, 2006). In analogy with the function of the neuronal munc13 proteins, munc13-4 might serve as an essential priming factor for secretory lysosomes in hematopoietic cells. Strong support for this idea derives from the findings that mutations in human munc13-4 cause familial hemophagocytic lymphohistiocytosis type 3 (FHL3), an autoimmune disease that is phenotypically related to GS2 (Feldmann *et al.*, 2003). Munc13-4 might have additional functions upstream of a role in the biogenesis of secretory lysosomes in CTLs (Menager *et al.*, 2007).

This chapter describes the method we used to search for proteins regulating release from secretory lysosomes using rab27a as bait. It is a modification of a procedure originally developed to screen for rab5 effectors (Christoforidis and Zerial, 2000). We also provide assays to analyze the properties and function of munc13-4 in a mast cell model.

2. Affinity Purification of rab27a Effector Proteins

2.1. Solutions

Lysis buffer: PBS containing 200 μM GDP, 5 mM $MgCl_2$,10 μg/ml DNase, 10 μg/ml RNase, 5 mM 2-mercaptoethanol, 10 μg/ml lysozyme, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 100 μM PMSF

Nucleotide exchange buffer (NE): 20 mM Na-HEPES, pH 7.5, 100 mM NaCl, 10 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothroitol (DTT) containing 10 μM GMP-PNP or 10 μM GDP (both Sigma)

Nucleotide stabilization (NS) buffer: 20 m*M* Na-HEPES, pH 7.5, 100 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* DTT, 10 μM GMP-PNP or 10 μM GDP

Elution buffer (EB): 20 m*M* Na-HEPES, pH 7.5, 1.5 *M* NaCl, 20 m*M* EDTA, 1 m*M* DTT, 1 m*M* GMP-PNP (for rab GDP column) or 5 m*M* GDP (for rab GTP-column)

Homogenization buffer (HB): 20 m*M* Na-HEPES, pH 7.5, 100 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* DTT, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 100 μM PMSF

2.2. Expression of GST-rab27a in *E. coli*

Human rab27a cDNA was cloned in the BamH1 site of pGEX2T (Neeft *et al.*, 2005). The plasmid encoding the fusion protein and as control pGEX plasmid is transformed into *Escherichia coli* strain BL21DE3 and grown on LB/agar plates containing 0.1 mg/ml ampicillin. A colony is grown at 30° in LB containing 0.1 mg/ml ampicillin, and after 8 h the culture is diluted 10 to 20 times and grown overnight at 30°. The next morning the culture is diluted 10 to 20 times and grown at 30° until OD600 ~0.6, which usually takes 2 to 3 h. Isopropyl-β-D-thiogalactopyranoside (IPTG) is added to 1 m*M*, and the culture is continued at the same temperature. After 4 h, 250-ml aliquots are harvested by centrifugation in a SLA-3000 rotor for 10 min at 4000×*g* and 4°. Pellets are washed with LB medium, snap frozen in liquid nitrogen, and stored at −80° until further use.

2.3. Isolation of GST-rab27a and guanine nucleotide loading

Frozen bacteria pellets are thawed and resuspended in 0.05 (culture) volume lysis buffer. The bacteria suspension is sonicated twice for 45 s on ice using a Branson Probe sonicator operating at 70% of maximum output. The homogenate is spun for 1 h at 4° at 100,000×*g* in a Ti45 rotor. The supernatant is next incubated with 0.5 ml prewashed (in PBS) GSH-sepharose 4B beads (Amersham) for 2 h under rotation at 4°. Beads are washed three times with PBS containing 100 μM GDP, 5 m*M* $MgCl_2$, and 5 m*M* 2-mercaptoethanol, and used immediately or stored overnight at 4° in the same buffer. For guanine nucleotide loading, beads are washed with NE buffer, and then incubated with this buffer containing 1 m*M* GMP-PNP for 30 min under rotation at room temperature (RT). This cycle is repeated twice, whereupon the beads are washed once with NS buffer and finally incubated with NS buffer containing 1 m*M* GMP-PNP for 20 min under rotation at RT. The above procedure is for loading GST-rab27a with a nonhydrolyzable GTP analog to screen for rab27a effectors with a putative function in secretion from lysosomes that bind to the active form. The same method is followed for GDP loading (except that GMP-PNP is replaced with GDP) in order to find proteins that bind to rab27a-GDP. This matrix

also serves as a negative control for proteins that are retrieved on the GTP-loaded rab27a. Before use in the assay, an aliquot of the beads is run on a 10% SDS-PAA gel together with a calibration curve of BSA to normalize the GST-rab27a.GDP, GST-rab27a.GMP-PNP, and GST input.

2.4. Preparation of pig spleen cytosol

All steps in the preparation of cytosol are done on ice in the cold room. A fresh pig spleen is diced and either immediately processed, or frozen in liquid nitrogen and stored at –80°. Tissue is immersed in two volumes of ice-cold HB, and homogenized using a Waring blender operating in 10-s time intervals and maximum speed until the tissue chunks are broken. The resulting suspension is subsequently homogenized in a Kinematica tissue homogenizer operating at 20 to 30% of maximum power. The sample is then centrifuged for 40 min at 4° and 10,000×*g* in a Sorvall SLA 3000 rotor. The supernatant is retrieved and centrifuged for 1 h at 4° and 100,000×*g* in a Ti45 rotor to generate a cytosol fraction. Cytosol is next dialyzed overnight against 1000 volumes of NS buffer using dialysis tubing with a 3500–molecular weight cut-off (Spectrumlabs). The retentate is centrifuged for 1 h at 4° and 100,000×*g*. Protein concentration in the cytosol supernatant is determined with the BCG assay (Pierce) and routinely amounts to 25 to 30 mg/ml.

2.5. Affinity isolation of cytosolic proteins on GST-rab27a.GMP-PNP beads

Beads are incubated with 15 ml of cytosol in the presence of 100 μM GMP-PNP under rotation at 4°. After 4 h, beads are washed twice with 2 ml NS buffer and 10 μM GMP-PNP, followed by two washes with 2 ml NS buffer, 250 mM NaCl, and 10 μM GMP-PNP, and two washes with 1 ml 20 mM Na-HEPES, pH 7.5, 250 mM NaCl, and 1 mM DTT. Bound proteins are eluted for 20 min at RT in a shaker with 20 mM Na-HEPES, pH 7.5, 1.5 M NaCl, 20 mM EDTA, 1 mM DTT, and 5 mM GDP. For elution of proteins bound to rab27aGDP, we replace GDP with 1 mM GMP-PNP. The elution is repeated once to increase the yield. Results of a typical affinity purification are shown in Fig.13.1A. Eluates are denatured for 5 min at 100° in reducing Laemmli buffer, and proteins are resolved on 6% SDS-PAA gels that are subsequently subjected to silver or Coomassie Brilliant Blue staining. Bands of interest are excised and in-gel digested using modified trypsin (Roche) in 50 mM ammonium bicarbonate. Digests are analyzed by nano-flow liquid chromatography–tandem mass spectrometry (LC-MS/MS), by using an electrospray ionization quadrupole time-of-flight mass spectrometer operating in positive ion mode. A nano-flow liquid chromatography system is coupled to the quadrupole time-of-flight,

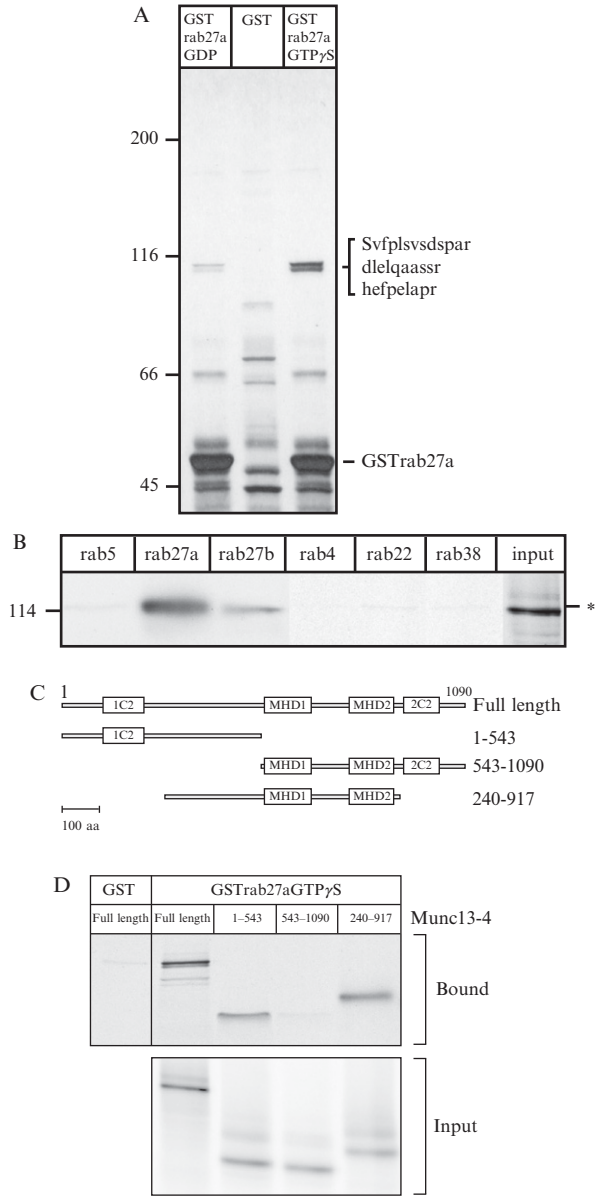

Figure 13.1 Characterization of munc13-4/rab27a complex on secretory lysosomes. (A) Affinity purification of munc13-4 from spleen on rab27a column. Spleen cytosol was incubated with GSH beads containing GST (negative control) and GST-rab27a charged with GDP or nonhydrolyzable GTP analog. Note the enrichment of 110-kDa protein on the GST-rab27aGMP-PNP beads. (B) Specific and direct binding of munc13-4 to rab27a. A panel of GMP-PNP–loaded GST-rab proteins was assayed for binding to ^{35}S-labeled munc13-4 produced in an *in vitro* transcription translation reaction. Note

essentially as described previously (Yatsuda *et al.*, 2003). Peptide mixtures are delivered to the system using a Famos autosampler (LC Packings) at 3 μl/min and trapped on an AquaTM C18RP column of 1 cm×100 μm (Phenomenex). After flow splitting down to 150 to 200 nl/min, peptides are transferred to an analytical column of 25 cm×50 μm (PepMap, LC Packings) in a gradient of acetonitrile (1%/min). Database searches are performed using Mascot software.

2.6. Comments

Although we used frozen pig spleen cytosol successfully, we get best results from freshly prepared material. Instead of cytosol, we also used 1% TX-100 lysates prepared from spleen. Since guanine nucleotide is not stably associated with most rab proteins in the presence of this concentration TX-100, we dilute the detergent in the binding reaction to 0.2%. For best results, we use the guanine nucleotide–loaded rab proteins and the dialyzed cytosol within 1 day after preparation. The preparative affinity isolations are done with an amount of GST-rab27a fusion protein that derived from 1 liter of *E. coli* culture immobilized on 0.5 ml GSH beads. We use two types of negative controls in the assay: (1) beads with the same amount of GST-rab27a fusion protein but now charged with GDP-nucleotide, and (2) beads with only GST.

3. Direct Binding Assay with Recombinant Proteins

To determine specificity of the candidate effector toward a collection of GST-rab proteins (Fouraux *et al.*, 2003; Neeft *et al.*, 2005), we employ a slightly modified binding assay in which the effector is produced as a

specificity in binding of munc13-4 to rab27. (C) Domain organization of munc13-4. Munc13-4 contains N-terminal and C-terminal C2 domains and two Munc13 homology domains (MHD) interspersed between them. This architecture is conserved in all munc13 proteins, and likely contains the minimal information required for function (Basu *et al.*, 2005; Stevens *et al.*, 2005). Indicated are munc13-4 truncations that were used for determining the rab27a binding domain and the requirements for cellular localization. (D) Binding domain of rab27a on munc13-4. Binding assay of ^{35}S-labeled His_6-munc13-4 truncations produced in an *in vitro* transcription translation reaction, and GMP-PNP–loaded GST-rab27a. From these experiments we inferred that the rab27a binding domain is situated between the first C2 and first MHD.

^{35}S-labeled polypeptide in a coupled *in vitro* transcription–translation reaction. This material is then used in a binding assay with GST-rab proteins.

3.1. Solutions

Binding buffer (BB): 50 m*M* Na-HEPES, pH 8.0, 150 m*M* NaCl, 0.05% Triton X-100, 5 m*M* 2-mercaptoethanol, 10 μM GMP-PNP
^{35}S methionine PRO-MIX (Amersham)
TNT T7 Quick master mix (Promega)

3.2. Constructs

pGEX-rab27a, pGEX-rab27b, pGEX-rab4a, pGEX-rab5a, pGEX-rab22, pGEX-rab38, pcDNA3.1HisB-munc13-4, pcDNA3.1HisB-munc13-4 (1–543), pcDNA3.1HisB-munc13-4(543–1090), pcDNA3.1HisB-munc13-4(240–917), pcDNA3.1HisB-munc13-4(Δ608–611)

3.3. Protocol

To determine specificity of the candidate effector toward rab proteins, we employ a slightly modified binding assay in which the effector is produced as a ^{35}S-labeled polypeptide in a coupled *in vitro* transcription–translation reaction. The ^{35}S-labeled effector protein is then incubated with a panel of GTP-loaded GST-rab proteins. In a typical reaction for five incubations, 1 μg of a T7-driven expression construct is mixed with 2 μl ^{35}S methionine PRO-MIX (Amersham) and 40 μl TNT T7 Quick master mix (Promega). Double-distilled H_2O is added to a final volume of 50 μl, and the mixture is incubated 1 h at 30°. In the meantime, beads containing immobilized, guanine nucleotide–charged GST-rab27a are washed once with BB.

Washed beads are then incubated for 2 h under rotation at 4° with 10 μl *in vitro* transcription–translation reaction product in 300 μl BB and 1 m*M* GMP-PNP. Beads are washed 4 times with BB, 0.2% Triton X-100, and 10 μM GMP-PNP. Bound protein is released by boiling for 5 min in reducing Laemmli buffer, resolved on a 10% SDS-PAA gel, and analyzed by phosphor imaging. Results of a specificity assay are shown in Fig. 13.1B.

3.4. Comment

The assay is used to establish or confirm guanine nucleotide–dependent binding to GST-rab27a. In addition, we employed the assay to map the rab27a binding domain using munc13-4 truncation constructs (Fig. 13.1C and D), and to analyze munc13-4 patient mutants for rab27a binding (Fig. 13.3C). For each binding reaction, we use 50-μl GSH beads and GST-rab27a isolated from a 50-ml bacteria culture.

4. Generation of an Antibody Against Munc13-4

A cDNA encoding munc13-4(904–1021) was generated by PCR and subcloned into the XhoI site of the bacterial expression plasmid pGEX4T3. The construct was transformed into *E. coli* BL21 (DE3) and grown on LB/agar plates containing 0.1 mg/ml ampicillin. A colony is grown at 30° in LB containing 0.1 mg/ml ampicillin, and after 8 h the culture is diluted 20 times and grown overnight at 30°. The next morning the culture is diluted 10 to 20 times and grown at 30° until OD600 ~0.6. IPTG is added to 1 m*M*, and the culture is continued at the same temperature. After 4 h, bacteria are harvested by centrifugation in a SLA-3000 rotor for 10 min at 4000×*g* and 4°. Pellets are washed with LB medium, snap-frozen in liquid nitrogen, and stored at –80° until further use. Frozen bacteria pellets are thawed, resuspended in 60 ml lysis buffer (see previous discussion), and broken in two sonication steps of 45 s on ice. The bacteria are centrifuged for 1 h at 4° at 100,000×*g*, and then the supernatant is retrieved and incubated with 0.5 ml GSH sepharose 4B beads (washed PBS) for 2 h under rotation at 4°. Unbound material is removed by centrifugation, and the beads are washed four times with 1 ml PBS, and 5 m*M* 2-mercaptoethanol. GST-munc13-4(904–1021) was eluted at 4° in two steps of 45 min, with each 0.5 ml of 50 m*M* Na-HEPES, pH 8.0, 25 m*M* GSH, and 5 m*M* 2-mercaptoethanol. The eluates are then combined and split into two equal fractions. One of these was boiled for 5 min in the presence of 0.1% SDS and 10 m*M* DTT (final concentrations), followed by addition of N-ethylmaleimide to 20 m*M* alkylate free sulfhydryl groups. Finally, the fusion proteins were dialyzed overnight at 4° against 1000 volumes of PBS. This procedure yielded ~1 mg of native and denatured GST-munc13-4(904–1021). Rabbits were then immunized with a mixture of the two forms to obtain antibodies against native and denatured protein. These antibodies were used to screen expression of munc13-4 in hematopoietic cell lysates, which revealed (among other things) high expression in the RBL-2H3 mast cell model and CD8-positive T cells (not shown).

4.1. Cell culture and transfection

4.1.1. Plasmids

pECFP-rab27a, pEYFP-munc13-4, pEGFP-munc13-4, pEGFP-munc13-4 (1–543), pEGFP-munc13-4(543–1090), pEGFP-munc13-4(240–917), pEGFP-munc13-4(Δ608–611)

4.1.2. Transfection of RBL cells and morphological assays

The antibodies against rab27a and munc13-4 are not good enough to detect the endogenous proteins by morphological methods. To determine localization of munc13-4 and requirements for membrane association, we expressed YFP of GFP-tagged munc13-4 constructs in RBL-2H3 cells for fluorescence microscopy. RBL-2H3 cells are cultured in DMEM, supplemented with 10% fetal calf serum, L-glutamine, and 50 μM 2-mercaptoethanol, and passed every 2 days. For transfection, 5×10^6 cells were harvested by trypsinization, resuspended in 250 μl DMEM, transferred to a 4-mm electroporation cuvette (BioRad), and mixed with 5 μg of plasmid DNA. A pulse of 1000 μF and 240 V was applied using a gene pulser (BioRad), and cells were immediately transferred to dishes containing prewarmed complete culture medium. With this method, ~5% of the cells are transfected. Using this protocol we found that munc13-4 and rab27a extensively colocalized on serotonin containing secretory lysosomes (Fig.13.2A), and that membrane localization of munc13-4 critically relied on the region containing the MHDs (see Figs. 13.2C and 13.3A). Higher transfection efficiencies of 20 to 30% are achieved with nucleofaction (Amaxa GmbH). In a typical protocol, 1×10^6 RBL-2H3 cells are resuspended in 100 μl T-buffer (Amaxa). The cell suspension and 1 μg plasmid DNA are transferred to the Amaxa cuvette and transfected with program X-001 in the Amaxa nucleofactor. Cells are transferred to 12 ml prewarmed culture medium and plated out into a six-well plate.

For immunofluorescence and imaging experiments, cells are grown on 10-mm #1 coverslips and used 1 day after transfection. Cells are washed once with PBS, fixed with 3% paraformaldehyde for 30 min, washed once with PBS, and incubated for 5 min with PBS containing 50 mM NH_4Cl. Fixed cells are washed once with PBS and incubated for 1 h in PBS containing 0.5% BSA and 0.1% saponin (block buffer). Incubation with a monoclonal serotonin antibody (DakoCytomation) is done for 1 h, followed by three 5-min washes with block buffer. Staining with appropriately labeled secondary antibodies is done for 1 h, followed by three 5-min washes with block buffer and two washes with PBS. Coverslips are mounted with 3 μl Moviol (Hoechst). Cells stably expressing YFP-munc13-4 were fixed with 2% paraformaldehyde in 0.1 M sodium phosphate buffer for immuno–electron microscopy. After fixative removal, the cells were embedded in 10% gelatin, and prepared for ultrathin cryosectioning and immunogold labeling (for detailed procedures, see Raposo *et al.*, 1997). Ultrathin cryosections were double labeled with a rabbit antibody against GFP, a mouse monoclonal antibody against the lysosomal membrane glycoprotein p80 (Bonifacino *et al.*, 1986), and various combinations of protein A gold particles. A rabbit anti-mouse Ig antibody (DAKO) was used as a bridging step in case of monoclonal antibodies. At the ultrastructural level, munc13-4 localized predominantly to the limiting membrane of

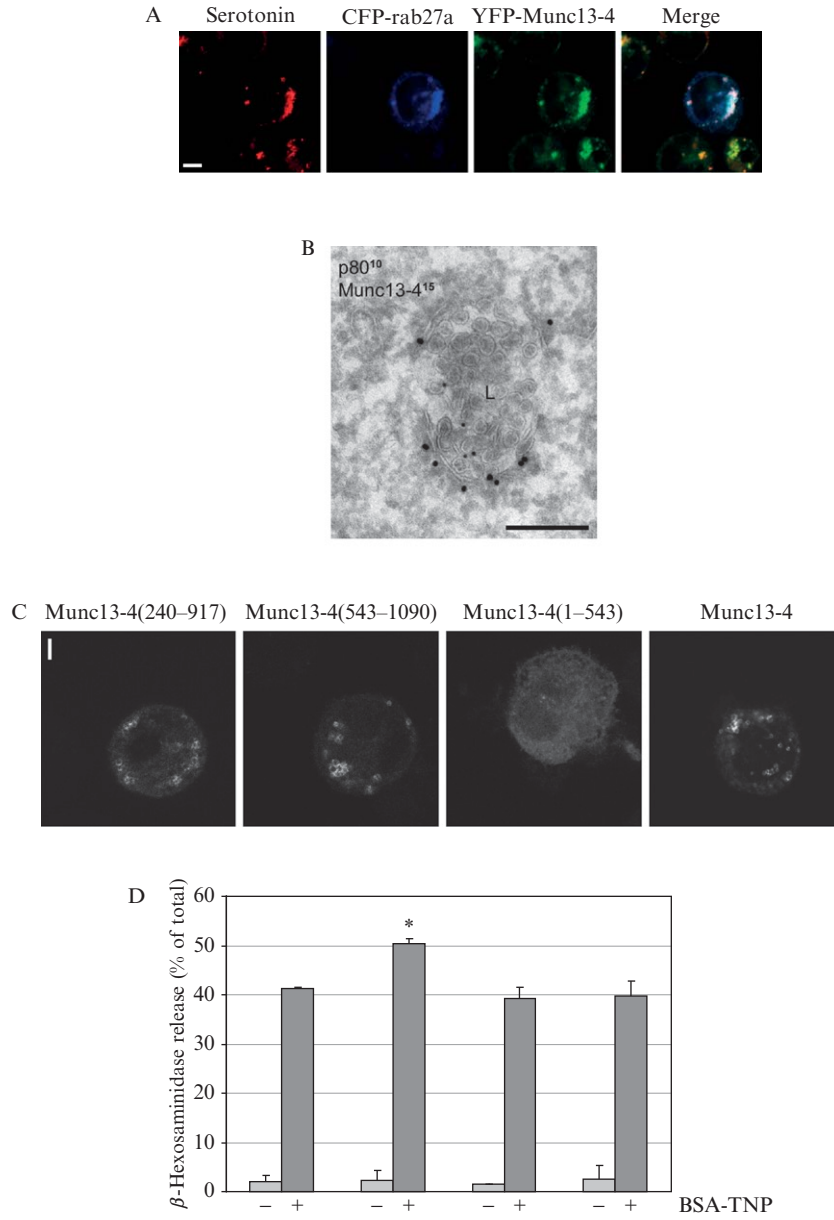

Figure 13.2 Localization of munc13-4/rab27a complex on secretory lysosomes. (A) Munc13-4/rab27a colocalize on granular structures in the cytoplasm. RBL-2H3 cells were transfected with pEYFP-munc13-4 (green) and pECFP-rab27a (blue), and labeled with an antibody against serotonin (red), a content marker of secretory lysosomes. The merged image of this triple-label experiment shows extensive colocalization of munc13-4/rab27a with the marker on discrete cytoplasmic granules. Bar denotes 5 μm.

a multivesicular compartment in which the lumenal structures were decorated with an antibody against the lysosomal membrane protein p80 (Fig.13.2B).

4.2. Comment

Some RBL-2H3 clones grow in suspension and poorly adhere to glass coverslips. For microscopy experiments, we coat glass coverslips with poly-L-lysine (Sigma) before seeding the cells.

5. Degranulation Assay in RBL-2H3 Cells

5.1. Reagents

DMEM + pen/strep + 1% FCS (secretion medium)
IgE anti-TNP mouse hybridoma supernatant
BSA
BSA-TNP (Sigma)
Triton X-100
p-nitrophenyl-N-acetyl-α-D-glucosaminide (Sigma)
0.1 *M* Na-carbonate buffer, pH 10

5.2. Plasmids

pEYFP-munc13-4, pEGFP, pEYFP-munc13-4, pEGFP-munc13-4 (543–1090), pEGFP-munc13-4(Δ608–611)

5.3. Method

We analyzed the function of munc13-4 in RBL-2H3 cells transfected with various munc13-4 constructs as described previously. Degranulation is induced by addition of IgE antibody and FcεR1 cross-linking with the antigen TNP-BSA. Lysosome release is then read out as β-hexosaminidase

(B) Munc13-4 localizes to the limiting membrane of multivesicular granules. Double labeling of YFP-munc13-4 (15 nm gold) and the lysosomal membrane glycoprotein p80 (10 nm gold) on cryosections prepared from RBL-2H3 transfectants. Bar denotes 200 nm. (C) Membrane localization of munc13-4 requires the MHDs. RBL-2H3 cells transfected with various GFP-tagged munc13-4 truncations were labeled with an antibody against serotonin. Note that only the truncations containing the region encompassing the MHDs localize to secretory lysosomes. Bar denotes 5 μm. (D) Munc13-4 is a positive regulator of secretory lysosome degranulation. RBL-2H3–expressing GFP-tagged munc13-4 constructs were assayed for their ability to release β-hexosaminidase in resting state and after stimulation. Full-length, munc13-4 enhanced antibody induced β-hexosaminidase secretion, while truncation mutants were without effect.

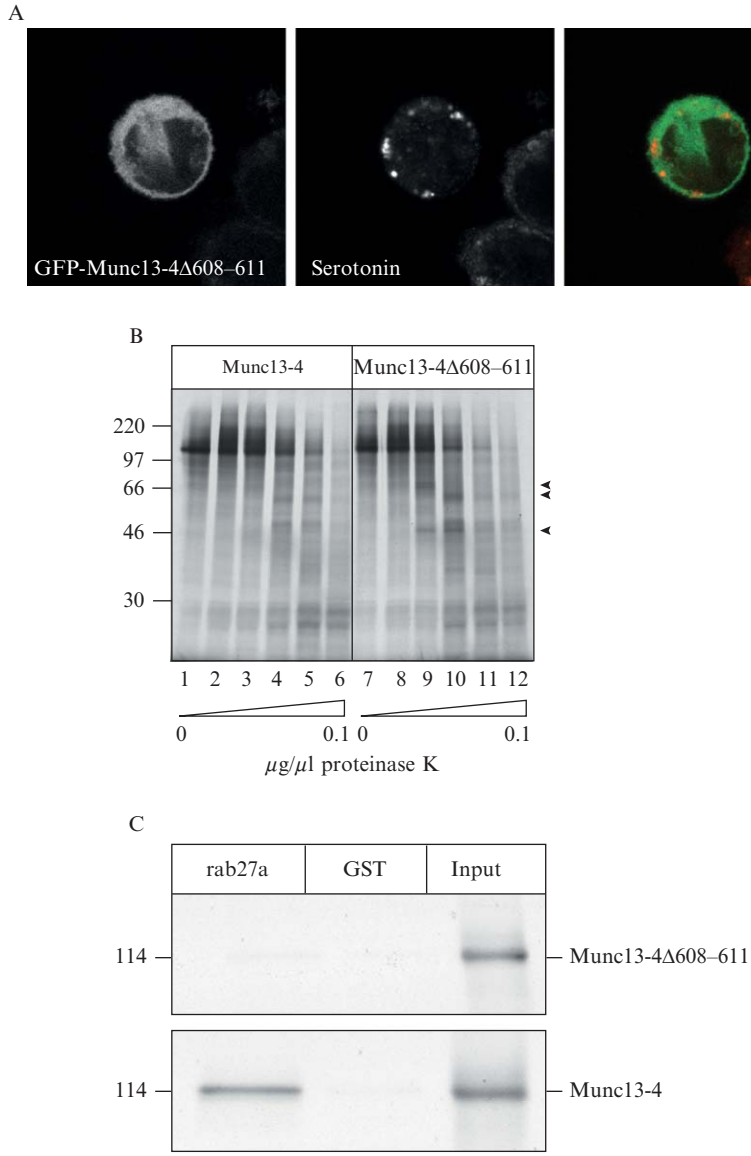

Figure 13.3 Analysis of FHL3 munc13-4Δ608–611 mutant. (A) Munc13-4Δ608–611 does not localize to secretory lysosomes. The FHL3 mutant (and control wildtype munc13-4) were transfected as GFP-tagged constructs in RBL-2H3 cells. Deletion of four amino acids in the first MHD creates a mutant that localizes to the cytoplasm, and is not recruited to serotonin containing secretory lysosomes. (B) Deletion of aa 608–611 causes misfolding of munc13-4. Conformational alterations of munc13-4Δ608–611 were investigated in a limited proteolysis assay. ^{35}S-labeled His$_6$munc13-4 were produced in a

accumulation in the medium. Transfectants are grown to a density of 70% and then scraped in 0.5 ml secretion medium. Next, 5×10^6 cells are pelleted, resuspended in 0.5 ml IgE anti-TNP hybridoma supernatant, and subsequently incubated at 37°. After 20 min, cells are washed with secretion medium and resuspended in 0.5 ml of this medium. Aliquots of 75 μl cell suspension are pipetted in a 96-well plate and coincubated for 1 h at 37° with 75 μl BSA-TNP (4 ng/ml) or the same concentration of BSA (negative control). The 96-well plate is centrifuged at 1000 rpm for 5 min, and 10 μl supernatant is transferred to a second 96-well plate containing 50 μl 2 m*M* p-nitrophenyl-N-acetyl-α-D-glucosaminide and 40 μl PBS. The incubation is run for 1 h at 37°, and stopped by addition of 150 μl 0.1 *M* Na carbonate buffer, pH 10. The absorbance at 405 nm is then determined in a plate reader. An aliquot of cells is solubilized in 0.2% TX-100 to determine the total cellular content of β-hexosaminidase, and to calculate the extent of degranulation. Figure.13.2D shows that transfection of wild-type munc13-4 enhanced degranlation, while two mutants were without effect.

5.4. Comments

Since transfection efficiency is low in RBL-2H3 cells, we use stable cell lines for degranulation experiments. Stable transfectants were selected in the presence of 1 mg/ml G418. Cells can also be activated with a combination of 1 μM ionomycin (Calbiochem) and 80 n*M* phorbol 12-myristate 13-acetate (Sigma).

6. Limited Proteolysis of ^{35}S-Labeled munc13-4

To determine whether point mutants or short truncations that are found in FHL3 cause conformational alterations to munc13-4, we use a limited proteolysis assay. The approach capitalizes on the principle that accessibility of protease target sites is dictated by the structure of a protein.

coupled transcription–translation reaction and submitted to limited proteolysis with protease K. The arrowheads denote differences between the digestion products of the wildtype and mutant proteins, showing that the mutation causes conformational alterations. (C) Deletion of aa 608–611 inhibits rab27a binding. Although the rab27a binding region is not in the MHD, it is necessary to determine whether rab27a binding is affected in the FHL3 mutant. GMP-PNP–charged GST-rab27a was used in a binding assay with ^{35}S-labeled His$_6$munc13-4Δ608–611. Note that deletion of aa 608–611 essentially precludes binding to rab27a, suggesting that the small deletion causes significant alterations to the overall conformation of munc13-4, which result in the failure to bind rab27a.

Mutations that cause conformational changes should also give rise to distinct proteolytic fragments when the sample is analyzed on a SDS-PAA gel. Indeed, the digestion products of the patient mutant munc13-4Δ608–611 are distinct from those of wildtype munc13-4 both for protease K (Fig. 13.3B) and for endoproteinase Glu-C (not shown).

6.1. Plasmids

pcDNA3.1HisB-munc13-4, pcDNA3.1HisB-munc13-4Δ608–611

6.2. Reagents

^{35}S methionine, 10 μCi/μl (Amersham Biosciences)
TNT T7–driven coupled *in vitro* transcription–translation system (Promega)
Proteinase K (Roche)
Endoproteinase Glu-C (Roche)
PMSF (Calbiochem)
Cycloheximide (Sigma)

6.3. Procedure

For each protease digestion, 1 μl (0.5 μg/μl) cDNA is mixed with 7 μl *in vitro* transcription–translation mixture, 1 μl ^{35}S methionine, and 1 μl distilled water, and incubated at 30°. After 1 h, cycloheximide is added to 1 m*M* to inhibit ongoing protein synthesis. The reaction is next transferred to ice and incubated with proteinase K (0.25, 1, 5, 25, and 100 μg/ml) or endoproteinase Glu-C (5, 25, 100, and 500 μg/ml). After precisely 15 min, PMSF is added to 2.5 m*M* to stop the reaction. Samples subsequently receive one volume two times reducing Laemmli buffer, and are boiled for 5 min prior to SDS PAGE.

6.4. Comment

Although protease concentrations need to be optimized for each protein protease combination, the indicated concentrations have given good results for a range of proteins.

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