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Munc13-4 Is an Effector of Rab27a and Controls Secretion of Lysosomes in Hematopoietic Cells

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Griscelli syndrome type 2 (GS2) is a genetic disorder in which patients exhibit life-threatening defects of cytotoxic T lymphocytes (CTLs) whose lytic granules fail to dock on the plasma membrane and therefore do not release their contents. The disease is caused by the absence of functional rab27a, but how rab27a controls secretion of lytic granule contents remains elusive. Mutations in Munc13-4 cause familial hemophagocytic lymphohistiocytosis subtype 3 (FHL3), a disease phenotypically related to GS2. We show that Munc13-4 is a direct partner of rab27a. The two proteins are highly expressed in CTLs and mast cells where they colocalize on secretory lysosomes. The region comprising the Munc13 homology domains is essential for the localization of Munc13-4 to secretory lysosomes. The GS2 mutant rab27aW73G strongly reduced binding to Munc13-4, whereas the FHL3 mutant Munc13-4Δ608-611 failed to bind rab27a. Overexpression of Munc13-4 enhanced degranulation of secretory lysosomes in mast cells, showing that it has a positive regulatory role in secretory lysosome fusion. We suggest that the secretion defects seen in GS2 and FHL3 have a common origin, and we propose that the rab27a/Munc13-4 complex is an essential regulator of secretory granule fusion with the plasma membrane in hematopoietic cells. Mutations in either of the two genes prevent formation of this complex and abolish secretion.

INTRODUCTION

Rab GTPases serve as important regulators of membrane transport in eukaryotic cells (Deneka *et al*., 2003b). Most rabs are ubiquitously expressed, but some have a more restricted distribution. For instance, rab27a is highly expressed in melanocytes and hematopoietic and other secretory cells (Tolmachova *et al*., 2004). Mutations causing loss of rab27a function in human result in defects of pigmentation (Bahadoran *et al*., 2001) and defects in the granule exocytosis pathway in cytotoxic T lymphocytes (CTLs) (Menasche *et al*., 2000). This rare autosomal recessive disease is called Griscelli syndrome type II (GS2) (Sanal *et al*., 2002).

Mutations in the genes encoding myosin-Va and the rab27a effector melanophilin also cause a pigmentation phenotype in human (Pastural *et al*., 1997; Menasche *et al*., 2003) and mice. These observations led to the identification of a ternary complex consisting of rab27a/melanophilin/myosin-Va that is essential for the normal function of melanosomes (Wu *et al*., 2002). A related complex containing rab27a/myRIP/myoVIIa seems to be important for melanosome localization in retinal pigment epithelium (Amraoui *et al*., 2002) and secretory granules in the PC12 pheochromocytoma cell line (Desnos *et al*., 2003).

Melanocytes and hematopoietic cells combine the functions of lysosomes and secretory granules into a hybrid organelle, the melanosome and secretory lysosome, respectively. Secretory lysosomes are particularly found in cells of the hematopoietic lineage, such as natural killer cells, CTLs, mast cells, dendritic cells, B cells, and neutrophils. They have an acidic lumenal pH and contain lysosomal enzymes. Nevertheless they are thought to be distinct from conventional lysosomes. First, stimulation of, for instance, the T-cell receptor complex on CD8-positive T-cells, or the high-affinity Fcε receptor on mast cells triggers the release of biological effectors such as granzymes and perforin in CTLs, and of serotonin and histamine in mast cells. Second, membrane repair that is typically executed by conventional lysosomes, or the release of conventional secretory products is not effected in a number of congenital diseases that selectively jeopardize secretory lysosome function (Blott and Griffiths, 2002). In spite of the relationship between melanosomes and secretory lysosomes, they use distinct machineries for fusion with the cell surface because mutations in myosin-Va and melanophilin do not cause the lethal cytotoxic activity that is associated with GS2. Accordingly, the regulatory function of rab27a in the lytic granule pathway of CTLs and Natural Killer cells depends on distinct effector proteins.

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We here identified Munc13-4 as a rab27a binding protein from spleen. Munc13-4 is highly expressed in several hematopoietic cells, including CTLs and mast cells. We describe the molecular features of the interaction and show that Munc13-4 is a positive regulator of secretory lysosome exocytosis. The importance of this interaction is shown by a patient FHL3 mutant (Feldmann *et al*., 2003), which cannot engage with rab27a in a complex on secretory granules of mast cells and is functionally inactive.

MATERIALS AND METHODS

Constructs

cDNAs encoding rab proteins, rabip4′, and rat Munc13-4 have been described previously (Koch *et al*., 2000; Fouraux *et al*., 2003). Melanophilin cDNA was a gift of Seji Torii (Gunma University, Maebashi City, Japan). Full-length human Munc13-4 cDNA was constructed from overlapping human expressed sequence tag (EST) clones (Invitrogen, Carlsbad, CA) and inserted into pCMV-SPORT6, pEYFP, pcDNA3, and pcDNA3.1His. Truncation constructs Munc13-4(aa 1–543), Munc13-4(aa 543-1090), and Munc13-4(aa 240–917) were generated by polymerase chain reaction (PCR) and subcloned in pEGFP, and pcDNA3.1His. The Munc13-4Δ608-611 mutant was made by site-directed mutagenesis by using QuikChange (Promega, Madison, WI) and cloned in pcDNA3.1His and pEGFP. cDNAs encoding human rab27a and rab27b were provided by Bill Gahl (National Institutes of Health, Bethesda, MD) and subcloned into pGEX, pEGFP, pECFP, and pcDNA3. The rab27aT23N, rab27aW73G, and rab27aQ78L mutants were created by site-directed mutagenesis with the QuikChange kit and recloned in pGEX and pEGFP. All tags were fused to the N terminus of cDNAs, and all synthetic cDNAs were verified by dye termination sequencing.

Antibodies

Antibodies against rat Munc13-4 were generated by immunizing rabbits with GSTmunc13-4 (aa 889-1088). An antibody against human rab27a was made in rabbits by immunization with GSTrab27a, and the rabbit antibody against green fluorescent protein (GFP) was purchased from BD Biosciences (Franklin Lakes, NJ). The monoclonal antibody (mAb) 5G10 against p80 was generously provided by Juan Bonifacino (National Institutes of Health). Monoclonal antibodies were obtained from the indicated sources; rab27a (BD Transduction Laboratories, Lexington, KY), rat CD63 (BD Biosciences PharMingen, San Diego, CA), serotonin (DakoCytomation California, Carpinteria, CA), EXPRESS (Invitrogen, Carlsbad, CA), and actin (MP Biomedicals, Irvine, CA). Conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Pull-Down Assays

Pig spleen cytosol was made as described previously (Deneka *et al*., 2003a). Glutathione *S*-transferase (GST)-rab proteins were produced in *Escherichia coli* BL21(DE3) grown at 30°C and immobilized on glutathione beads as per the vendors' instructions. Immobilized GST-rab proteins were loaded with guanine nucleotide and incubated for 4 h with spleen cytosol at 4°C as described previously (Christoforidis *et al*., 1999). Beads were washed, and bound cytosolic proteins were eluted with 1.5 M NaCl, 20 mM EDTA, 5 mM GDP, 1 mM dithiothreitol (DTT), 20 mM HEPES, pH 7.5. For columns of GDP-loaded rab27a, GDP in the elution buffer was replaced by 1 mM guanosine 5′-*O*-(3-thio)triphosphate (GTPγS). Eluates were boiled in Laemmli sample buffer and resolved on 6% SDS-PAA gels and silver-stained. ^{35}S-labeled rab effectors were synthesized in T7 and SP6-driven reticulocyte lysate systems (Promega) and used in pull-down assays with GTPγS-charged GSTrab proteins (Christoforidis *et al*., 1999). Bound proteins were eluted with 25 mM reduced glutathione and resolved on 10% SDS-PAA gels.

Mass Spectrometry

Bands of interest were excised and in-gel digested using modified trypsin (Roche Diagnostics, Indianapolis, IN) in 50 mM ammonium bicarbonate. Digests were analyzed by nanoflow 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 liquid chromatography system was coupled to the quadrupole time-of-flight essentially as described previously (Yatsuda *et al*., 2003). Peptide mixtures were delivered to the system using a Famos autosampler (LC Packings, Sunnyvale, CA) at 3 μl/min and trapped on an AquaTM C18RP column of 1 cm × 100 μm (Phenomenex, Torrance, CA). After flow splitting down to 150–200 nl/min, peptides were transferred to an analytical column of 25 cm × 50 μm (PepMap; LC Packings) in a gradient of acetonitrile (1%/min). Database searches were performed using Mascot software (www.matrixscience-.com).

Cells and Transfection

HeLa cells were transfected with FuGENE 6 (Roche Diagnostics), and RBL-2H3 cells were transfected by electroporation (960 μF, 240 V). Stable RBL-2H3 transfectants were generated by selection in 0.6 mg/ml G418 and fluorescence-activated cell sorting. The Jurkat human T-cell line, the Raji human B cell line, the U937 human macrophage cell line, the YT.2C2 human Natural Killer cell line, the K562 human erythroleukemia cell line, the CTLL-2 mouse cytotoxic T-cell clone, the D1 mouse dendritic cell line (Winzler *et al*., 1997), and the 32D mouse myeloid cell line were provided by Paul Coffer and Jeanette Leusen (University Medical Center Utrecht). The RBL-2H3 rat mast cell line was provided by Magda Deneka (Laboratory for Molecular Cell Biology, London, United Kingdom), and primary human melanocytes were generously given by Nico Smit (Department of Dermatology, Leiden University Medical Center, Leiden, The Netherlands). The BLM and 530 human melanoma cell lines were provided by Guido Swart (Department of Biochemistry, UMC Nijmegen, The Netherlands).

Microscopy

Transfected RBL-2H3 cells were grown for 2 d in DMEM supplemented with 10% fetal calf serum (FCS), 20 mM HEPES, L-glutamine, and antibiotics, and subsequently adhered to poly-L-lysine–coated coverslips. Cells were fixed with 3% paraformaldehyde and subjected to confocal scanning fluorescence microscopy as described previously (Deneka *et al*., 2003a). RBL-2H3 cells stably expressing YFP-munc13-4 were fixed with 2% paraformaldehyde in 0.1 M sodium phosphate buffer for immunoelectronmicroscopy. After fixative was removed, the cells were embedded in 10% gelatin and prepared for ultrathin cryosectioning and immunogold labeling (Raposo *et al*., 1997). Ultrathin cryosections were double immunolabeled with different combinations of antibodies and protein A gold particles. A rabbit anti mouse immunoglobulin antibody (DakoCytomation California) was used as a bridging step in case of monoclonal antibodies.

RBL-2H3 Degranulation Assay

Transfected RBL-2H3 cells were grown to 60% confluence, harvested by scraping, and washed. Typically, 1×10^6 cells were incubated for 20 min at 37°C with 0.1 ml of IgE anti-TNP hybridoma supernatant. Cells were washed and next incubated for 1 h at 37°C with 10 ng/ml bovine serum albumin (BSA)-TNP in RPMI 1640 medium containing 1% FCS. Nonspecific release was determined in the same manner, except that the second incubation was done with BSA instead of BSA-TNP. The total amount of β-hexosaminidase was determined in lysates prepared from cells that were extracted with 0.2% Triton X-100. To quantitate the extent of degranulation, 50 μl of culture supernatant was incubated for 1 h at 37°C with 50 μl of 2 mM *p*-nitrophenyl-*N*-acetyl-α-d-glucosaminide in 0.05 M citrate buffer, pH 4.5. The enzyme reaction was stopped by addition of 150 μl of 0.05 M carbonate buffer, pH 10.0, and the absorbance at 405 nm was measured. The percentage of β-hexosaminidase release was calculated relative to the total amount of β-hexosaminidase.

Limited Proteolysis Experiments

To assay for conformational differences between wild-type Munc13-4 and Munc13-4 mutants, we used limited proteolysis followed by analysis of digestion products by SDS-PAGE. For limited proteolysis experiments, we produced ^{35}S-labeled Munc13-4 and Munc13-4Δ608–611 in an in vitro transcription translation at 30°C. Reactions were stopped after 60 min with 1 mM cycloheximide and cooled to 4°C. Proteases were then added at 0.1, 0.025, 0.005, 0.001, and 0.00025 μg/μl (proteinase K) or 0.5, 0.1, 0.025, 0.005, and 0.001 μg/μl (endoproteinase Glu-C) for 15 min. Digestions were stopped with phenylmethylsulfonyl fluoride and immediately boiled in Laemmli buffer and loaded on a 10% SDS-PAA gel.

Miscellaneous Methods

To determine the expression level of Munc13-4, cells were washed with phosphate-buffered saline and lysed in Laemmli sample buffer containing 50 mM DTT. Samples were subjected to SDS-PAGE and analyzed by Western blot by using a rabbit antibody against Munc13-4, a mAb against rab27a, and monoclonal antibodies against actin or tubulin (loading controls).

RESULTS

Identification of Munc13-4 as rab27a Binding Protein in Spleen

To search for rab27a effectors in hematopoietic cells, we performed preparative pull-down assays with GSTrab27a charged with nonhydrolyzable GTPγS, or with GDP, and spleen cytosol. We used splenic tissue because it contains a sizeable pool of T-cells and is highly enriched in rab27 (Seabra *et al*., 1995). Proteins that were specifically bound to

the columns were eluted with EDTA and high salt. EDTA chelates Mg^{2+} and thereby impairs guanine nucleotide binding to rab27a, which causes dissociation of effector proteins that bind in a nucleotide-dependent manner. Eluates were resolved by SDS-PAGE and were silver-stained. We found a 115-kDa protein that ran as a doublet on 6% gels and that was preferentially enriched on GSTrab27a-GTPγS (Figure 1A). The two bands were collectively cut from the gel, trypsinized, and analyzed by LC-MS/MS. We obtained amino acid sequences of three peptides that uniquely identified them as the pig ortholog of rat Munc13-4. Because Munc13-4 contains several putative phosphorylation sites, it is possible that one of the bands is a phosphorylated form. Alternatively Munc13-4 might be partially degraded, or the second band might represent a distinct protein. We assembled a full-length human Munc13-4 cDNA from human EST clones and ligated this in pCMV-SPORT6.

Munc13-4 is a distant relative of the C2 domain-containing Munc13 proteins that with exception of a ubiquitously expressed Munc13–2 splice variant are predominantly expressed in the nervous system. They serve as priming factors of glutaminergic synaptic vesicles and are essential for synaptic transmission (Rosenmund *et al*., 2002). Munc13-4 differs in several ways from Munc13-1, Munc13-2, and Munc13-3 (Koch *et al*., 2000). First, it is expressed mainly outside of the nervous system. Second, Munc13-4 does not contain the diacylglycerol-binding C1 domain that is present in the other Munc13 proteins and is required for the function of Munc13-1 (Rhee *et al*., 2002). Third, Munc13-4 is appreciably smaller than the other Munc13 proteins that have molecular masses >200 kDa.

The GS2 Mutant rab27aW73G Binds Poorly to Munc13-4

Given that Munc13-4 interacted specifically with the active form of rab27a, it qualifies as putative effector. To determine the specificity of the interaction and to rule out that Munc13-4 was bound to rab27a via a cytoplasmic linker protein, we incubated GTPγS-charged GSTrab27a beads with ^{35}S-labeled human Munc13-4. Both rab27a and rab27b bound directly to Munc13-4. Rab27b, however, seemed to bind to a lesser extent than rab27a, possibly because of a higher exchange rate or less efficient GTPγS loading. This conclusion is supported by the observation that rab27b also bound less efficiently to melanophilin. We also found that rab3a bound to Munc13-4 in the assay (our unpublished data). Because rab3a is not expressed outside the nervous system (Schlüter *et al*., 2002), we did not pursue this interaction further. None of the other rab proteins that we tested bound to Munc13-4 (Figure 1B). We confirmed that the interaction was guanine nucleotide specific, because the GTP hydrolysis-deficient mutant rab27aQ78L recapitulated binding, whereas the GDP-bound rab27aT23N mutant did not.

The observation that rab27a interacted directly with Munc13-4 allowed us to evaluate the consequences of rab27a mutations found in GS2 patients with respect to their binding to Munc13-4. We selected the homozygous missense mutation 217T>G because it leads to a single amino acid change, giving rise rab27aW73G, which shows a relatively mild GS2 phenotype (Menasche *et al*., 2000, 2002; Sanal *et al*., 2002). The other known GS2 mutations either introduce frame shifts or premature stop codons that are less useful for interaction studies. rab27aW73G is known to retain the ability to bind GTP and its intrinsic hydrolysis rate is reduced to the same extent as in rab27aQ78L. Nevertheless, its interaction with melanophilin is severely impaired (Menasche *et al*., 2002). Quantitation of Figure 1C showed that binding of ^{35}S-labeled human Munc13-4 to rab27aW73G was threefold reduced compared with wild-type rab27a and just above background binding to rab27aT23N. In agreement with this finding, we also observed by confocal immunofluorescence microscopy that rab27aW73G did not colocalize with Munc13-4 (Figure 6). The control binding experiment with melanophilin confirmed that rab27aW73G did not interact with the melanosomal effector.

Munc13-4 and rab27a Are Highly Expressed in a Subset of Hematopoietic Cells

Although Munc13-4 is a ubiquitous protein, it is highly expressed in a specific set of tissues (Koch *et al*., 2000), whereas rab27a is preferentially found in secretory cells (Tolmachova *et al*., 2004). We next determined the comparative expression pattern of Munc13-4 and rab27a in hematopoietic cell lines and melanocytes, especially because the function of these cells is mostly compromised in GS2. As positive control for the size of Munc13-4, we transfected Munc13-4 in HeLa cells (Figure 2A). As shown in Figure 2, Munc13-4 and rab27a were coexpressed in many of the cell lines. We observed high expression of the two proteins in the CTLL-2 (CTL) cell line, the RBL-2H3 mast cell line, and in myeloid (32D) cells. Importantly, although human melanocytes contained the highest level of rab27a expression in all of the cell lines that we tested, we did not detect Munc13-4 in these cells. To rule out that this was an oddity of the primary melanocytes, we also analyzed the expression of Munc13-4 in two human melanoma cell lines. As shown in Figure 2B, both the metastasing BLM and the nonmetastasing 530 cell lines did not express Munc13-4. Thus, Munc13-4 seemed not to be expressed in melanocytes. Control experiments with transfected rat and human Munc13-4 cDNAs revealed that the Munc13-4 antibody recognized human and rat Munc13-4 with the same affinity (our unpublished data).

Munc13-4 and rab27a Colocalize on Secretory Lysosomes of Mast Cells

Because the Western blot in Figure 2 showed that rab27a and Munc13-4 were highly expressed in mast cells, we next determined the intracellular distribution of the two proteins in this cell line. Mast cells belong to the immune system and constitute a typical example of hematopoietic cells with secretory lysosomes. They release lysosomal enzymes and biologically active mediators such as cytokines, serotonin, and histamine upon encountering specific antigen (Metcalfe *et al*., 1997). Although the mAb against rab27a detected the endogenous protein in mast cells (our unpublished data), the signal was to weak to be of use for double- and triple-label immunofluorescence experiments, whereas the antibody against Munc13-4 did not yield reliable signals for the endogenous protein by microscopical methods. We therefore expressed YFP-Munc13-4 and CFP-rab27a at low levels in RBL cells and compared their localization with respect to marker proteins. As shown in Figure 3, little if any Munc13-4 was found in the cytoplasm, whereas Munc13-4 and rab27a extensively colocalized with CD63, a marker for secretory lysosomes and multivesicular late endosomes (Kleijmeer *et al*., 2001). Because CD63 predominantly localizes on secretory lysosomes in mast cells (Puri *et al*., 2003), we concluded that Munc13-4 and rab27a mainly colocalized on this organelle. Occasionally these structures were localized in a narrow zone immediately below the plasma membrane. Munc13-4 and rab27a also colocalized with p80, another secretory lysosome marker in mast cells and with serotonin (Bonifacino *et al*., 1986; Bonifacino *et al*., 1999). Munc13-4 did not colocalize with the endoplasmic reticulum marker pro-

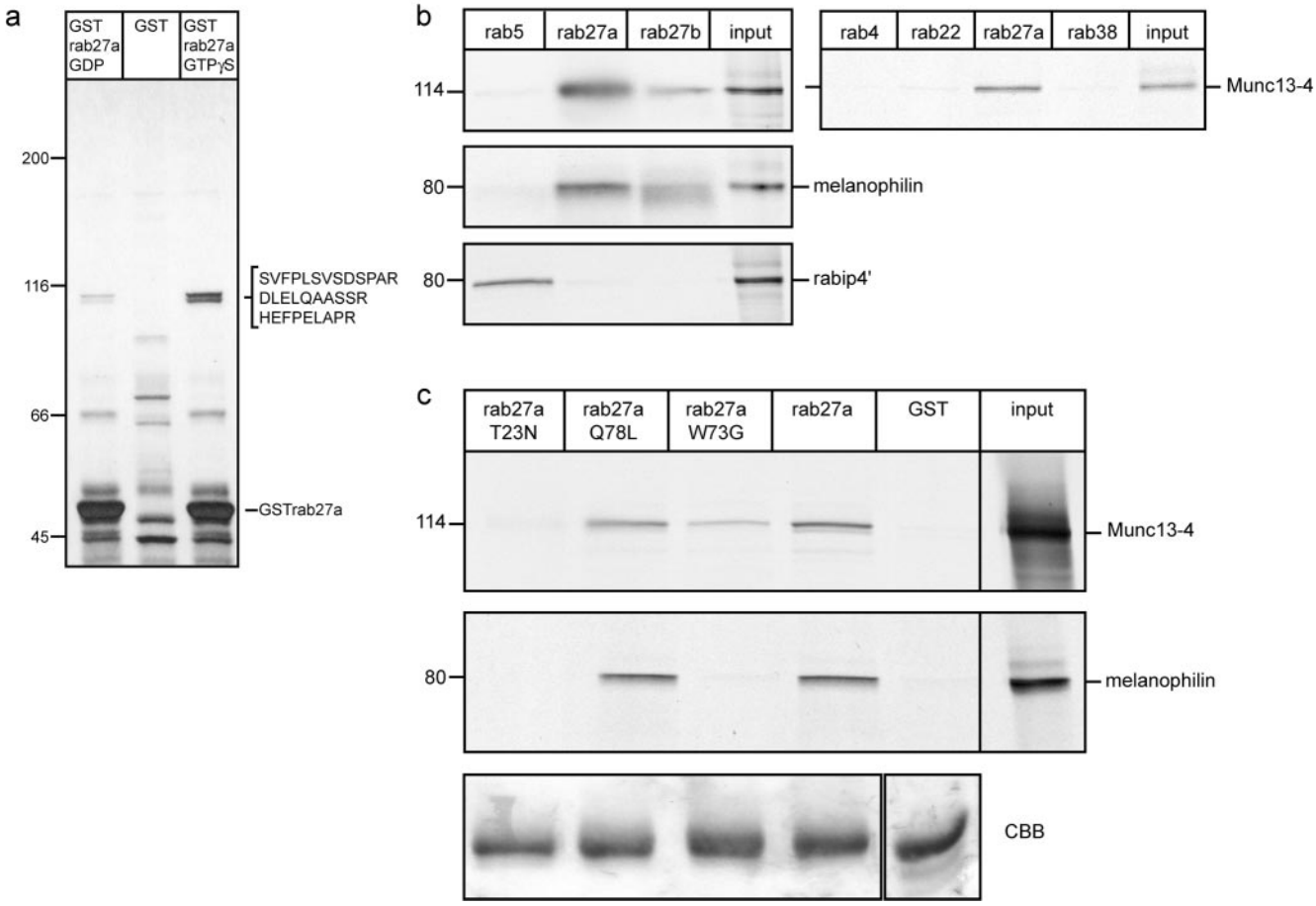

Figure 1. (A) Identification of Munc13-4 as a rab27 binding protein in spleen. Silver-stained 6% SDS-PAA gel showing preferential isolation of a 115-kDa doublet on rab27a-GTPγS beads. The indicated peptides were identified by LC-MS/MS. Direct and specific binding of ^{35}S-labeled Munc13-4 to rab27. GTPγS-charged GST rab proteins were incubated with ^{35}S-labeled Munc13-4. Bound protein were resolved on 10% SDS-PAA gels and visualized by phosphorimaging. (B) Functionality of rab27 fusion proteins was confirmed with melanophilin, and rabip4′, an effector of rab5. A GST fusion of the GS2 patient mutant rab27aW73G was loaded with GTPγS and incubated ^{35}S-labeled Munc13-4. As positive controls, we used rab27aQ78L and wild-type rab27a, both charged with GTPγS. As negative control the GDP mutant rab27aT23N was used. (C) Bound protein analyzed as in B and quantitated by phosphorimaging, rab27aW73G bound 3 times less Munc13-4 than wild-type rab27a.

tein disulfide isomerase and the Golgi marker GM130 (our unpublished data).

Ultrastructural Localization of Munc13-4

To determine the localization of Munc13-4 at higher resolution, we performed double-label immunoelectron microscopy on ultrathin cryosections prepared from RBL-2H3 cells transfected with YFP-Munc13-4 (Figure 4). The majority of Munc13-4, detected with an anti-GFP antibody, was observed in large vacuolar organelles with internal membranes and vesicles. The labeling for Munc13-4 was primarily found associated with the limiting membrane of these structures and occasionally with the plasma membrane (our unpublished data). Serotonin (Figure 4A) and p80 (Figure 4B) localized on the internal vesicles of the vacuolar organelles, indicating that the latter represented bona fide secretory lysosomes. Importantly, Munc13-4 was essentially absent from the internal membranes. In some cells, we observed the presence of Munc13-4 in the area of the Golgi complex, although not particularly enriched in the Golgi proper (our unpublished data).

Binding Site of rab27a on Munc13-4

To investigate the requirements for rab27a binding to Munc13-4, we generated truncation mutants of Munc13-4 (Figure 5A) and cloned these in pcDNA3.1His and pEGFP-C plasmids for binding and localization experiments. ^{35}S-labeled His-tagged Munc13-4 truncations were produced in in vitro transcription translation reactions and tested for their ability to bind to GTPγS-loaded GSTrab27a (Figure 5B) in a pull-down assay. As shown in Figure 5, rab27a binding to Munc13-4 required the N-terminal half of the protein. Removal of the C2 domains in Munc13-4(aa 240–917) did not affect binding to rab27a, showing that both C2 domains are dispensable for the interaction with rab27a. Because Munc13-4(aa 543-1090) lost the ability to bind rab27a, the binding experiments with truncation constructs suggested that the region of Munc13-4 comprising aa 240–543 is necessary for binding to rab27a.

Localization of Munc13-4 on Secretory Lysosomes Required Munc13 Homology Domains (MHDs)

We next determined the requirements for Munc13-4 localization to the secretory lysosomes. Because effectors of rab

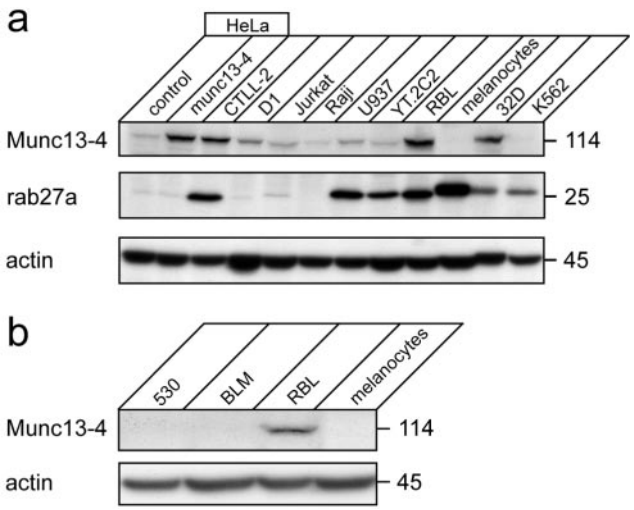

Figure 2. Expression of Munc13-4 and rab27a in hematopoietic cells. Detergent lysates were prepared from the indicated cell lines, resolved by 12.5% SDS-PAGE, and analyzed by Western blot by using antibodies against Munc13-4, rab27a, and actin (loading control). Munc13-4pcDNA3 was transfected in HeLa cells and served as size control. (A) Note that the CTL cell line (CTLL-2), the mast cell line (RBL), and the myeloid cells (32D) contain high levels of Munc13-4 and rab27a, whereas Munc13-4 was not detected in primary human melanocytes. (B) Western blot of lysates from human melanoma cell lines 530 and BLM showing that these cell lines did not express Munc13-4.

proteins are generally recruited by the GTP-bound form to the membrane where the small GTPase resides, we first assessed whether rab27a was important for the localization of Munc13-4. Thus, we transfected GFP-tagged rab27a constructs with His-tagged Munc13-4 in RBL-2H3 cells and analyzed the localization of the proteins by immunofluorescence microscopy (Figure 6A). In agreement with the results shown in Figure 3, GFP-rab27a and GFP-rab27aQ78L colocalized to a large extent with His-Munc13-4 on secretory lysosomes. Neither the GS2 mutant rab27aW73G that bound poorly to Munc13-4 nor rab27aT23N that did not interact with Munc13-4 colocalized with His-Munc13-4. Whereas GFP-rab27aW73G seemed to localize to amorphous cytoplasmic structures that did not label for serotonin and *trans*-Golgi network markers (our unpublished data). GFP-rab27aT23N was typically distributed to the cytoplasm. Importantly, His-Munc13-4 was associated with secretory lysosomes irrespective of the guanine nucleotide status of transfected rab27a. These results suggested that rab27a is not essential for Munc13-4 localization to secretory lysosomes. Alternatively, although less likely, endogenous rab27a might be sufficient to recruit transfected Munc13-4 to secretory lysosomes.

To discriminate between these two possibilities, we transfected GFP-tagged truncations of Munc13-4 in RBL cells and analyzed their localization by confocal fluorescence microscopy. Western blots of detergent lysates prepared from transfected cells showed that the expression level of the different Munc13-4 constructs was similar (our unpublished data). Secretory lysosomes were visualized by labeling for serotonin. As shown in Figure 6B, expression of the N-terminal half of Munc13-4(aa 1–543) containing the first C2 domain and the rab27a binding domain yielded a cytoplasmic localization that did not colocalize with secretory lysosomes, as evidenced by the mutually exclusive distribution with serotonin. The C-terminal half of Munc13-4(aa 543-1090) containing the two MHDs and the second C2 domain, however, localized to secretory lysosomes. The Munc13-4 construct lacking both C2 domains (aa 240–917) also localized to serotonin-containing organelles, showing that the C2 domains are not essential to secretory lysosome localization. Collectively, the fluorescence microscopy experiments with rab27a mutants and Munc13-4 truncations showed that the region containing the MHDs is essential for the specific localization, whereas rab27a binding is not.

The FHL3 Mutation Munc13-4Δ608-611 Is Mislocalized

A recent study showed that mutations in Munc13-4 are responsible for FHL3 (Feldmann *et al.*, 2003). The lytic granules of CTLs isolated from FHL3 patients seem to dock normally at the immunological synapse. However, they do not fuse with the plasma membrane and fail to release their lytic contents. Although the majority of the reported FHL3 patients had frame shifts or nonsense substitutions in the N-terminal half of Munc13-4, one of them had a short in-frame deletion of four amino acids in MHD1. Because we found that the central part of Munc13-4 containing the MHDs is of critical importance for its localization to secretory lysosomes, we further investigated the Munc13-4Δ608-611 mutant. GFP-tagged Munc13-4Δ608-611 was transfected in RBL cells, and its localization was assessed by confocal fluorescence microscopy. As shown in Figure 7A, Munc13-4Δ608-611 failed to associate with the secretory lysosomes as evidenced by the lack of colocalization with the markers p80 and serotonin. Importantly, the staining patterns of p80 and serotonin in cells transfected with Munc13-4Δ608-611 were similar as in the nontransfected cells. This observation showed that the mutant did not cause gross morphological alterations to secretory lysosomes and suggested that Munc13-4 is not involved in the biogenesis of secretory lysosomes.

Although we found that rab27a binding required the N-terminal half of Munc13-4, adjacent to but not containing MHD1, it is possible that deletion of amino acids 608–611 might cause structural alterations that affect conformation elsewhere in the protein. We therefore next addressed whether Munc13-4Δ608-611 retained the ability to bind rab27a. As shown in the input lanes of Figure 7B, Munc13-4Δ608-611 was produced to the same extent as wild-type Munc13-4. In contrast to wild-type Munc13-4, however, the deletion mutant did not bind to rab27a. Because the deleted amino acids are not part of the rab27a binding region, it is likely that the deletion alters the conformation or folding of Munc13-4. To address this question we performed limited proteolysis experiments of ^{35}S-labeled wild-type Munc13-4 and Munc13-4Δ608-611. Reproducible changes in the degradation pattern of Munc13-4Δ608-611 were seen with proteinase K (right) and endoproteinase Glu-C (left) as indicated by the closed arrow heads (Figure 7C, right). We concluded from these experiments that deletion of the 608Val-Gln-Arg-Ala611 sequence in the FHL3 mutant altered the conformation of Munc13-4 that correlated with the lack of binding to rab27a.

Munc13-4 Is a Positive Regulator of Degranulation in RBL-2H3 Cells

To determine whether the localization of Munc13-4 to secretory granules and the interaction with rab27a was functionally relevant, we generated stable RBL-2H3 cell lines expressing GFP, YFP-Munc13-4, GFP-Munc13-4(aa 543-1090), and Munc13-4Δ608-611. To minimize the risk of overexpres-

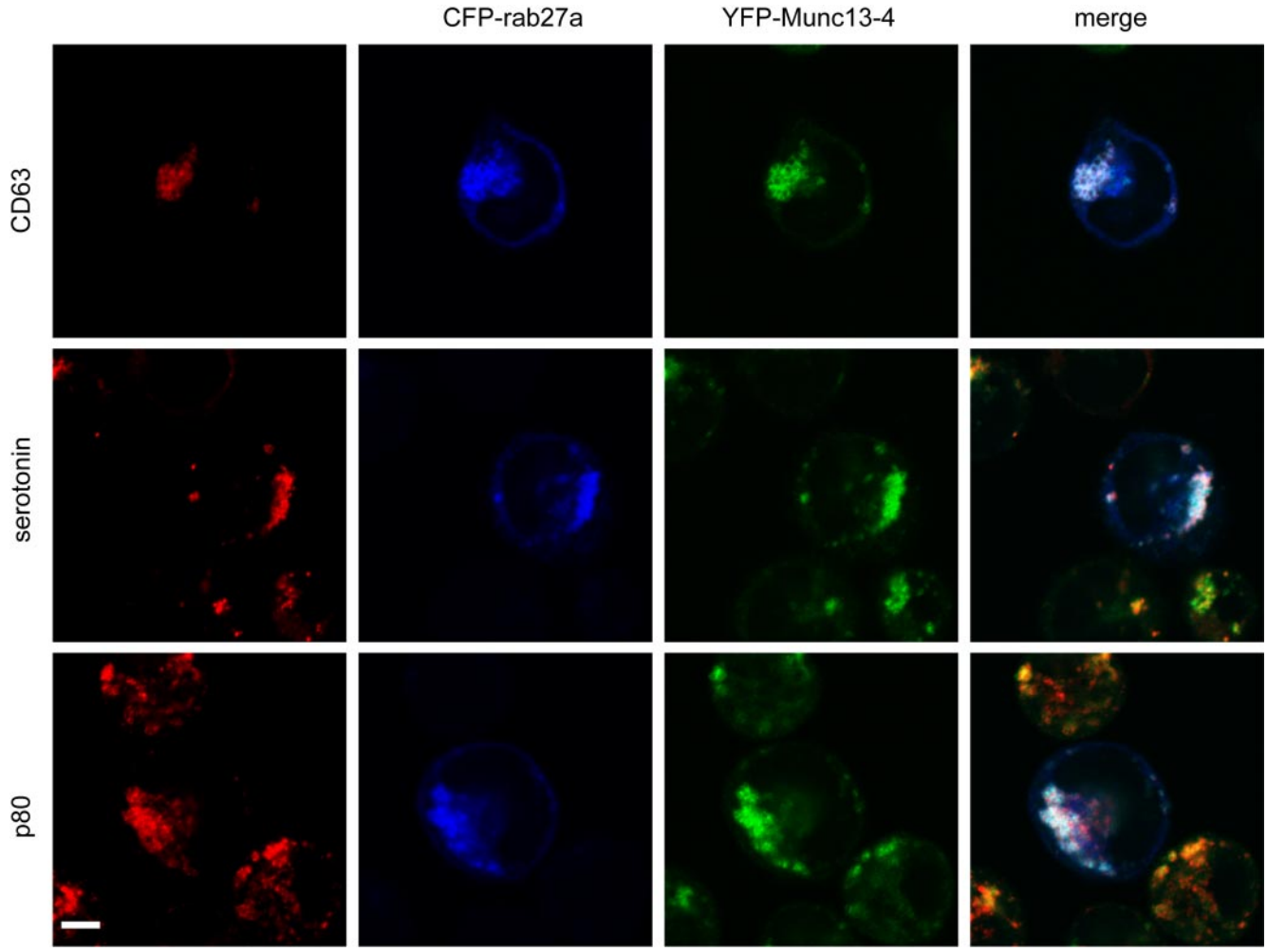

Figure 3. Rab27a and Munc13-4 colocalize on secretory lysosomes in mast cells. RBL-2H3 cells were transfected with YFP-Munc13-4 and CFP-rab27a and labeled with monoclonal antibodies against CD63, p80, and serotonin (red). The triple-labeled merged images show that CFP-rab27a (blue) and YFP-Munc13-4 (green) extensively colocalized on the secretory lysosomes that are decorated with antibodies against the markers as evidence by the white merged signals. Bar, 5 μm.

sion artifacts, we selected cells with low levels of Munc13-4 constructs to analyze their effects on degranulation of secretory lysosomes. Transfectants contained similar levels of the constructs as measured by Western blot with a GFP antibody (our unpublished data), whereas the extent of overexpression was slightly above that of endogenous protein as detected with a Munc13-4 antibody (Figure 8). Cells were then incubated with anti TNP IgE followed by TNP-bovine serum albumin, and released β-hexosaminidase was measured in the medium. BSA served as a control for nonspecific release, which was $<$3% of total β-hexosaminidase, irrespective of the transfected cDNA. In transfected cells expressing GFP, 40% of cellular β-hexosaminidase was released after TNP-bovine serum albumin stimulation. Moderate overexpression of Munc13-4 enhanced β-hexosaminidase release by 25% compared with the GFP-transfected control. In contrast, overexpression of neither the Munc13-4 mutant lacking the first C2 domain and the rab27a binding region nor the FHL3 patient mutant with the short deletion in MHD1 affected β-hexosaminidase secretion. Thus, Munc13-4 mutants that fail to interact with rab27a or do not localize to secretory lysosomes do not have an effect in the assay. Presumably the level of overexpressed mutants is too low to inhibit the function of endogenous Munc13-4. Together, these results suggest that Munc13-4 has a positive regulatory role in the stimulated release of secretory lysosome content into the medium and that both the rab27 binding domain and the secretory lysosome binding domain are required for its function.

DISCUSSION

We here report the isolation of Munc13-4 as a major and direct binding protein of rab27aGTP. We described its localization to secretory lysosomes in mast cells, and we elucidated the requirements for its binding to rab27a and for the association with membranes. We also showed that Munc13-4, but not mutants that fail to bind to rab27a, or do not localize to secretory granules, regulates secretory lysosome function in mast cells. Munc13-4 was originally identified by data base searching with a conserved motif present in Munc13 proteins of different species (Koch *et al.*, 2000). Munc13-4 lacks the N-terminal extension, and the C1 domain that is present in other members of the Munc13 family. For that reason, Munc13-4 membrane localization is likely regulated in a different manner than for the other Munc13

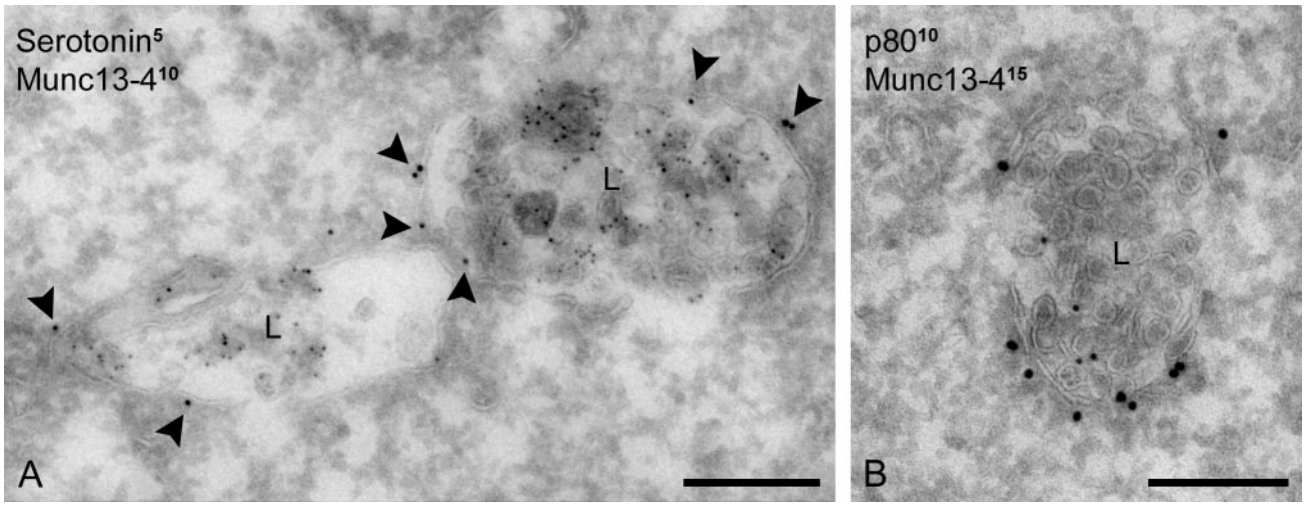

Figure 4. Ultrastructural localization Munc13-4. Ultrathin cryosections of RBL-2H3 cells stably expressing Munc13-4-YFP immunolabeled with polyclonal anti-GFP. Immunoelectron micrograph showing the presence of YFP-Munc13-4 (10-nm protein A gold particles) and antiserotonin (5 nm) in secretory lysosomes (L). Gold particles for Munc13-4 (arrowheads) are restricted to the limiting membrane (A). Double immunolabeling showing colocalization of p80 (10 nm) and YFP-Munc13-4 (15 nm) in lysosome (L) (B). Bar, 200 nm.

proteins. Indeed, fluorescence microscopy and immunoelectronmicroscopy showed that transfected Munc13-4 is predominantly localized to secretory lysosomes, whereas for instance Munc13-1 is evenly distributed between the cytoplasm and the plasma membrane when expressed in bovine chromaffin cells (Ashery *et al*., 2000).

Because Munc13-4 is a peripheral membrane protein, it likely dissociated from the membranes during subcellular fractionation, allowing for its isolation from a cytosol fraction. Others recently identified Munc13-4 as a rab27-GTP binding protein in platelets (Shirakawa *et al*., 2004). Using subcellular fractionation, these authors found, however, that Munc13-4 predominantly localized in the cytoplasm, whereas a second pool of Munc13-4 was associated with rab27a on the plasma membrane. The requirements for membrane localization of Munc13-4 are distinct from what is usually seen with rab effector proteins. Activation of a rab protein by a guanine nucleotide exchange factor leads to recruitment of cytoplasmic effector proteins to the compartment on which the rab resides (Deneka *et al*., 2003b; Pfeffer, 2003). This condition can be enhanced by overexpression of a wild-type or GTP hydrolysis-deficient mutant of the rab protein, whereas overexpression of a GDP mutant causes rab effectors to localize in the cytoplasm. Neither of these phenotypes were observed for Munc13-4 and rab27a. Transfected Munc13-4 associated with secretory lysosomes in the absence of cotransfected rab27a as well as in the presence of any of the rab27a constructs, irrespective of whether they represented GDP-bound or GTP-bound forms. In agreement with this, Munc13-4 (aa1–543) that contained the major rab27a binding site was not localized on secretory lysosomes but instead seemed to have a cytoplasmic distribution. These observations show that the C-terminal half of the protein containing the MHDs is the principal determinant of Munc13-4 association with secretory lysosomes.

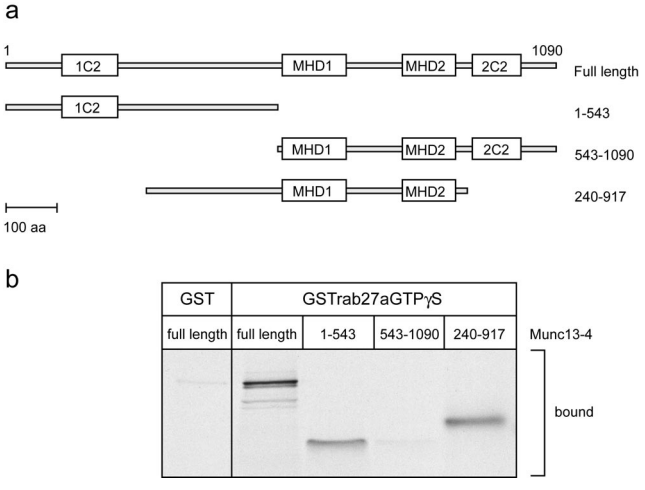

Figure 5. (A) Binding domain of rab27a on Munc13-4. Domain organization of Munc13-4 and truncation constructs (A). Binding of ^{35}S-labeled His-Munc13-4 truncations to GTPγS charged GST-rab27a was done as described under *Materials and Methods*. (B) Analysis of the Munc13-4 truncation mutants indicated that the rab27a binding domain is within the region comprising aa 240–543.

A number of rab27a effectors have been described that either belong to the C2 domain containing synaptotagmin-like protein (Slp) family or to the Slac family (Slp homologues lacking C2 domains) of which Melanophilin and MyRIP are typical representatives. Interaction of rab27a with Slac and Slp proteins critically depends on an N-terminal Slp homology domain (Kuroda *et al*., 2002) that is related to the motif in rabphilin-3A required for binding to rab3 (Ostermeier and Brunger, 1999). In this respect, Munc13-4 is a representative of a new class of rab27a effectors. Munc13-4 interacted preferentially with rab27a in the GTP-bound form. In addition, Munc13-4 binding required the Trp73 residue in rab27a because the substitution with Gly impaired the interaction. Trp73 is part of an invariant hydrophobic triad that is strictly conserved in all rab GTPases and that has been shown to be a determinant of effector recognition by different rab subfamilies (Merithew *et al*., 2001). Thus, even though the Munc13-4 structure and the precise residues involved in the interaction with rab27a remain to be established, it is likely that binding will follow in part related principles as have been defined for the interaction

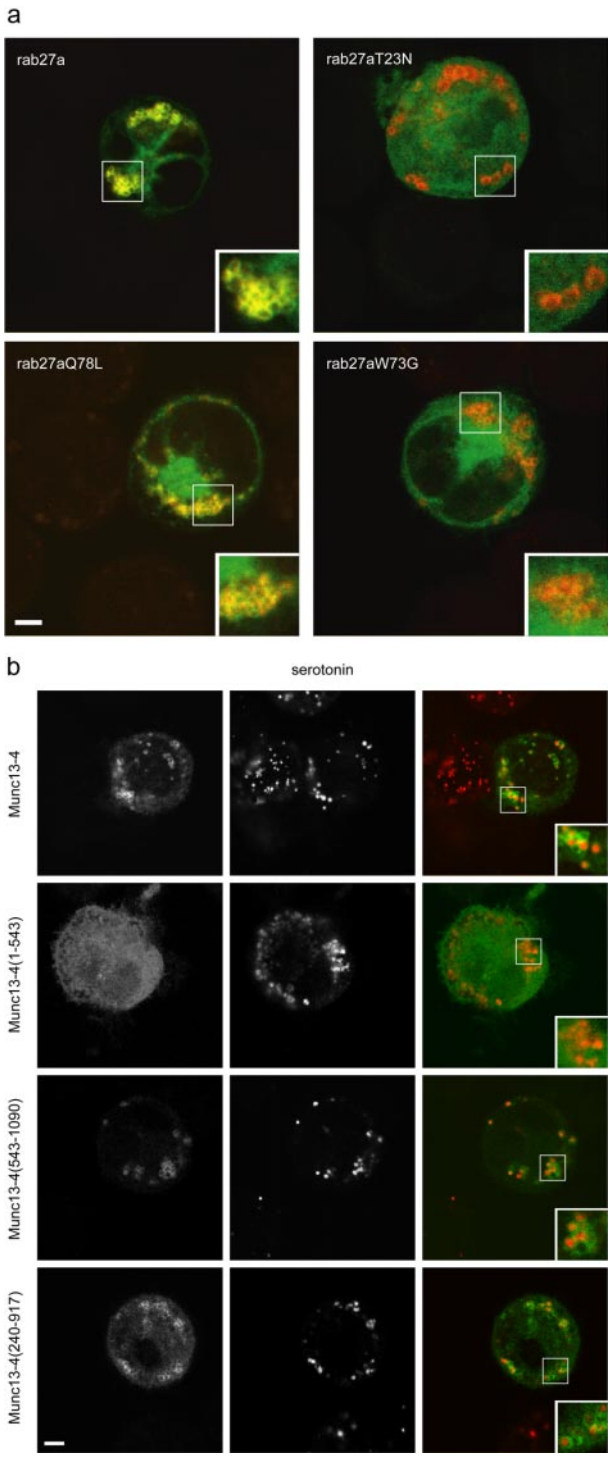

Figure 6. Localization of Munc13-4 on secretory lysosomes required MHDs. GFP-tagged rab27a (green) constructs were cotransfected with His-tagged Munc13-4 in RBL-2H3 cells. Fixed cells were labeled with the EXPRESS antibody for His-Munc13-4 (red). Wild-type rab27a and rab27aQ78L extensively colocalized on the limiting membrane of secretory lysosomes. The rab27aT23N mutant was diffusely distributed throughout the cytoplasm, whereas Munc13-4 is still targeted to secretory lysosomes. Unlike Munc13-4, the GS2 mutant rab27aW73G that poorly bound Munc13-4 did not localize to secretory lysosomes. (A) Transfection of GFP-tagged Munc13-4 truncation constructs (green) in RBL cells. (B) Cells were fixed and labeled with an antibody against serotonin (red). With the exception of Munc13-4(aa 1–543) that included the rab27a binding domain, the truncations localized to secretory lysosomes. Bar, 5 μm

between melanophilin and rab27a. The biological correlate for this idea is provided by the fact that the GS2 mutation rab27a-W73G affects both CTL and melanocyte functions that depend on different rab27a effectors.

A clue to the function of Munc13-4 with respect to rab27a was suggested by the finding that mutations in Munc13-4 are the cause for FHL3 (Feldmann *et al*., 2003; Yamamoto *et al*., 2004). CTLs of FHL3 patients lack functional Munc13-4 mRNA, and deficiency of Munc13-4 results in defective CTL granule secretion. Interestingly, although cytotoxic granules polarize normally and dock correctly at the immunological synapse, they fail to fuse with the plasma membrane (Feldmann *et al*., 2003). Earlier work established that CTL granules of rab27a-deficient GS2 patients do not dock at the immunological synapse (Menasche *et al*., 2000). The implication of these morphological phenotypes is that Munc13-4 might act downstream of rab27a in granule secretion; however, neither direct nor indirect binding of Munc13-4 to rab27a was established in these studies (Feldmann *et al*., 2003). 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 (cf. below). In agreement with this idea, we found at a low level of overexpression that Munc13-4 enhanced stimulus-dependent release of secretory lysosome contents. Earlier studies in which Munc13-1 was massively overexpressed in bovine chromaffin cells documented that a member of the Munc13 family enhanced large dense core vesicle exocytosis (Ashery *et al*., 2000).

Although Munc13-4 is a binding partner of rab27a, FHL3 patients do not have a pigmentation phenotype (Feldmann *et al*., 2003). This observation implies that the proper distribution of, and pigment release from, melanosomes is independent of Munc13-4 because it is not expressed in melanocytes. Although distinct in outcome, this situation is mechanistically reminiscent to the tissue-specific phenotype seen in Griscelli syndrome type 3 that is caused by mutations in melanophilin (Menasche *et al*., 2003). In contrast to rab27a mutations that affect both CTL and melanocyte functions, melanophilin mutations only affect melanocytes. This simply reflects the absence of melanophilin expression in CTLs. The selective disease phenotypes seen upon inactivating mutations of the rab27a effectors melanophilin and Munc13-4 provide a clear example of the paradigm that a single rab protein may exert multiple functions through interactions with distinct effector proteins in different cell types.

The tissue distribution of Munc13-4 is not limited to hematopoietic cells. It was shown before that Munc13-4 is strongly expressed in mucous goblet cells and alveolar type II cells in the lung (Koch *et al*., 2000), arguing for a more general function of Munc13-4 in tissues with secretory activity. Similarly, rab27a is more ubiquitously expressed than originally perceived (Tolmachova *et al*., 2004). It is likely therefore that GS2 and FHL3 patients may suffer from additional afflictions that were not noticed because they are not limiting health as much as CTL and Natural Killer cell malfunction. The restricted exocytosis phenotype seen in GS2 and FHL3 patients is therefore caused either by additional tissue-specific proteins of the rab27a effector network or by the absence of redundant factors that can compensate for the loss of rab27a and Munc13-4 function.

The closest homolog of rab27a is rab3 that together with rab26, rab37, and rab8 forms a subfamily of the rab branch of small GTPases (Pereira-Leal and Seabra, 2000). Evidence is now emerging that distinct rab proteins of the same subfamily may perform related functions through the use of the

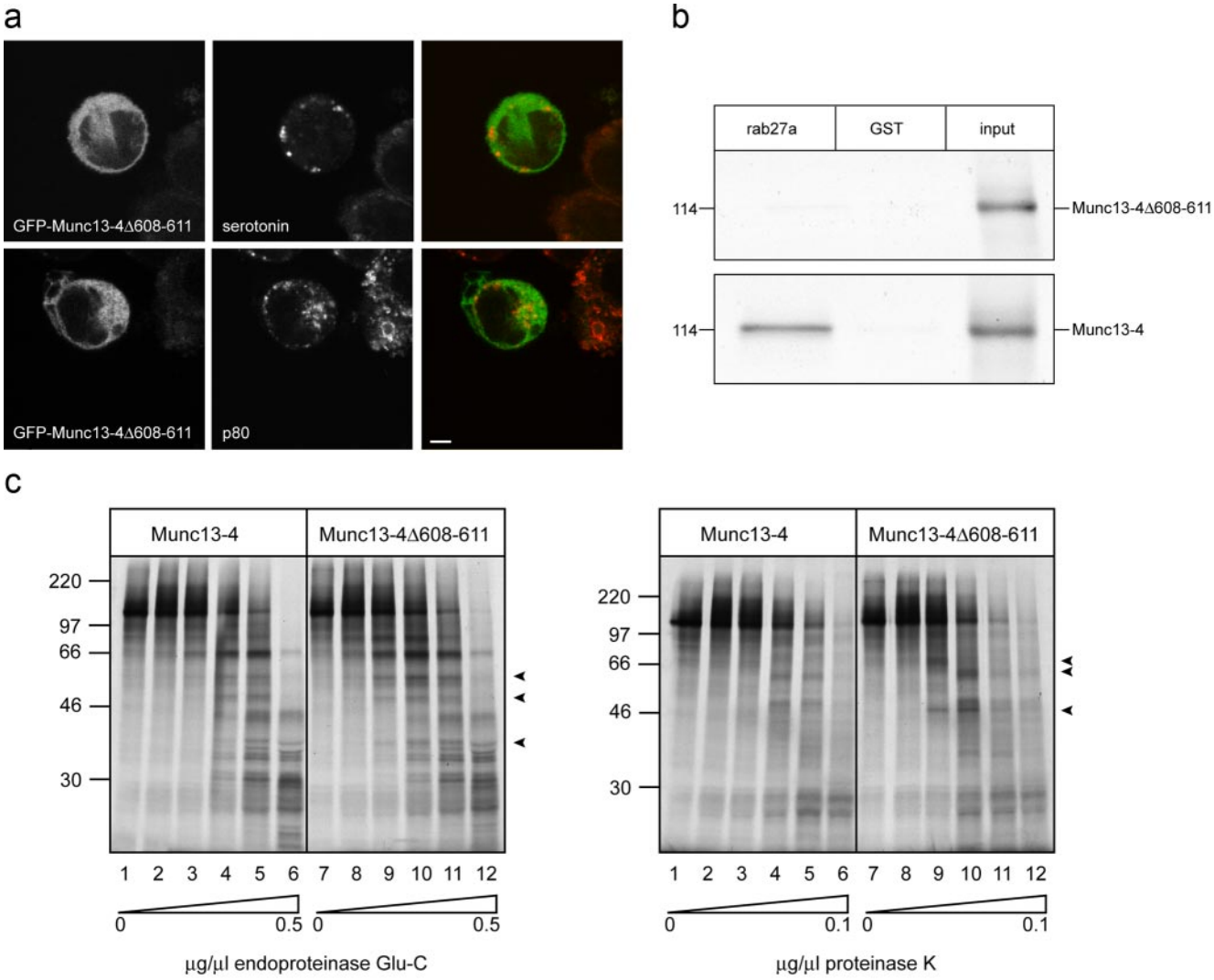

Figure 7. The Munc13-4Δ608-611 is mislocalized and does not bind rab27a. Transfection of GFP-tagged Munc13-4Δ608-611 (green) in RBL cells. Fixed cells were labeled for the granule markers p80 and serotonin (red). (A) Munc13-4Δ608-611 failed to be targeted to the secretory lysosomes. Bar, 5 μm. (B) Binding assay of rab27a-GTPγS with ^{35}S-labeled Munc13-4Δ608-611 showing that the deletion mutant does not bind rab27a. (C) Limited proteolyis of ^{35}S-labeled wild-type Munc13-4 (lanes 1–6) and Munc13-4Δ608-611 (lanes 7–12) with 0 (lanes 1 and 7), 0.001 (lanes 2 and 8), 0.005 (lanes 3 and 9), 0.025 (lanes 4 and 10), 0.1 (lanes 5 and 11), and 0.5 (lanes 6 and 12) μg/μl endoproteinase Glu-C (left), or with 0 (lanes 1 and 7), 0.00025 (lanes 2 and 8), 0.001 (lanes 3 and 9), 0.005 (lanes 4 and 10), 0.025 (lanes 5 and 11), and 0.1 (lanes 6 and 12) μg/μl proteinase K (right). Closed arrow heads denote alterations in sensitivity of Munc13-4Δ608-611 to the two proteases.

same effector proteins as was shown for rab27a and rab3 (Fukuda *et al*., 2004), or rab5a and rab22 (Kauppi *et al*., 2002). For rab3a and rab27a, this idea is strengthened by the appealing parallels between the immunological and neuronal synapses. Nevertheless, it is clear that a unifying concept for rab27a function in the immunological synapse and for rab3a function in the neuronal synapse does not exist. rab27a is absolutely essential for the release of secretory lysosome contents as testified by patients with GS2, whereas rab3 is not essential for synaptic vesicle exocytosis per se, as was recently shown in a complete genetic analysis of neuronal rab3 function (Schlüter *et al*., 2004). A second difference between rab3 and rab27a function relates to the fact that rab27a binds directly to Munc13-4, whereas neuronal Munc13-1 does not interact with rab3 (Betz *et al*., 2001). Third, synaptic vesicles release neurotransmitter contents within milliseconds after Ca^{2+} influx. Secretory lysosomes in CTLs first need to be transported to the plasma membrane in response to target cell recognition. Thus, synaptic vesicle exocytosis is a much faster process that likely requires specialized machinery not present in hematopoietic cells.

How might rab27a and Munc13-4 regulate release of secretory lysosome contents? The originally identified bona fide Munc13 proteins consist of two modules. A highly divergent N terminus that is not present in Munc13-4 and a generally conserved C-terminal part, required for synaptic vesicle priming. The domain organization of the C terminus is conserved in Munc13-4, and in Munc13-1 it contains interaction sites for putative Munc13 effectors, including syntaxin-1 (Brose *et al*., 2000). Given that Munc13-4 contains a region that is homologous to the syntaxin-1 binding domain of Munc13-1, we anticipate that Munc13-4 controls the activity of SNARE complexes that likely consist of different partners in the various cell types of the hematopoietic lin-

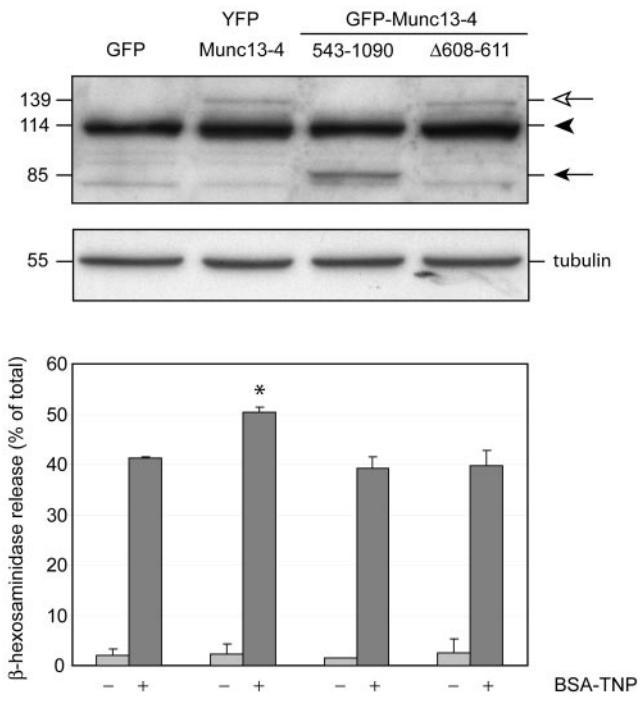

Figure 8. Munc13-4 is a positive regulator of degranulation. Western blot of lysates prepared from stable RBL-2H3 transfectants expressing GFP, YFP-Munc13-4, GFP-Munc13-4(aa 543-1090), and Munc13-4Δ608-611. Expressed constructs were detected with antibodies against Munc13-4, and tubulin (loading control). Arrowhead denotes endogenous Munc13-4, open arrow YFP-Munc13-4 and GFP-Munc13-4Δ608-611, and closed arrow GFP-Munc13-4(aa 543-1090). Transfectants were stimulated with IgE/TNP-bovine serum albumin, and degranulation was measured as release of β-hexosaminidase into the medium. Results are calculated as percentage of total β-hexosaminidase present in the cells. Nonspecific release of β-hexosaminidase was measured in the absence of TNP-bovine serum albumin and was <3% for the four cell lines. Moderate overexpression of YFP-Munc13-4 enhanced secretion significantly above control GFP-expressing cells (Student's *t* test, $p < 0.005$). Data are means of quadruplicates ± SE.

eage. Indeed, several candidate SNARE and Munc18 proteins have already been identified in platelets (Chen *et al*., 2000), macophages (Pagan *et al*., 2003), and mast cells (Paumet *et al*., 2000; Martion-Verdeaux *et al*., 2002; Puri *et al*., 2003) and have been shown to be involved in lysosomal secretion. We hypothesize that FcεR signaling in mast cells (Blank and Rivera, 2004) or T-cell receptor signaling in CTLs, activates a downstream rab27a guanine nucleotide exchange factor. As a result of which rab27a is converted into the active state, allowing it to associate with Munc13-4 on secretory granules and to concentrate Munc13-4 in a rab27a domain on the granule. Rab27a-dependent tethering of the granule to the plasma membrane would then place Munc13-4 in proximity of the fusion machinery. Assuming that Munc13-4 has a related role as the C terminus of Munc13-1 in promoting the open conformation of syntaxin (Richmond *et al*., 2001), release of the Munc18 clamp initiates SNARE pairing and fusion of the granule with the plasma membrane (Rizo and Sudhof, 2002). GTP-hydrolysis of rab27a causes dissociation of the tethering complex and of the rab27a/Munc13-4 complex and thereby reduces the local concentration of Munc13-4 below a level required for its positive regulatory function on formation of the core SNARE complex. Irrespective of the precise mechanism, our results suggest that the rab27a/Munc13-4 complex is an essential regulator of a yet unknown SNARE complex that mediates granule fusion with the plasma membrane in hematopoietic cells. Mutations in either rab27a or Munc13-4 that prevent formation of this complex therefore lead to the inability of CTLs to release their contents, as seen in Griscelli and FHL3 patients.

While this manuscript was under revision, Sasaki *et al*. submitted a paper reporting that Munc13-4 is a functional partner of rab27a in the RBL-2H3 mast cell line. This study confirms our results that Munc13-4 localizes to secretory lysosomes and that it acts as a positive regulator of mast cell degranulation (Goishi *et al*., 2004).

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