Identification of SNAREs Involved in Synaptotagmin VII-regulated Lysosomal Exocytosis*

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Ca2+-regulated exocytosis of lysosomes has been recognized recently as a ubiquitous process, important for the repair of plasma membrane wounds. Lysosomal exocytosis is regulated by synaptotagmin VII, a member of the synaptotagmin family of Ca2+-binding proteins localized on lysosomes. Here we show that Ca2+-dependent interaction of the synaptotagmin VII C2A domain with SNAP-23 is facilitated by syntaxin 4. Specific interactions also occurred in cell lysates between the plasma membrane t-SNAREs SNAP-23 and syntaxin 4 and the lysosomal v-SNARE TI-VAMP/VAMP7. Following cytosolic Ca2+ elevation, SDS-resistant complexes containing SNAP-23, syntaxin 4, and TI-VAMP/VAMP7 were detected on membrane fractions. Lysosomal exocytosis was inhibited by the SNARE domains of syntaxin 4 and TI-VAMP/VAMP7 and by cleavage of SNAP-23 with botulinum neurotoxin E, thereby functionally implicating these SNAREs in Ca2+-regulated exocytosis of conventional lysosomes.

Conventional lysosomes have been identified recently as the major intracellular compartment that undergoes Ca2+-triggered exocytosis in non-specialized secretory cells (1–3). This pathway is utilized by many cell types to reseal plasma membrane wounds (4, 5) and is also subverted by the intracellular parasite Trypanosoma cruzi in order to invade mammalian cells (6, 7). Previous studies showed that synaptotagmin (Syt) VII, a ubiquitously expressed member of a family of putative Ca2+ sensors for membrane fusion (8, 9), is involved in the regulation of lysosomal exocytosis and of membrane repair (2, 4, 5). Syt VII-deficient mice show defects in cell resealing and develop a form of autoimmune myositis, with inflammation and increased collagen accumulation in the skin and skeletal muscle (5).

Synaptotagmins contain two C2 domains in their cytosolic region, which can bind phospholipids in response to Ca2+, as well as components of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes (10). SNARE proteins are thought to be key mediators of all intracellular membrane fusion events (11). The most extensively characterized SNARE complex is the one involved in the exocytosis of synaptic vesicles in neurons. This complex consists of synaptobrevin/VAMP2 (vesicle-associated membrane protein 2) on the vesicle membrane, and syntaxin 1 and SNAP-25 (synaptosome-associated protein of 25 kDa) on the plasma membrane (12). Syntaxin 1 and VAMP 2 each contribute one α-helical domain and SNAP-25 two domains to a parallel four-helix coiled-coil bundle, which has been proposed to provide the force necessary to bring the opposing bilayers together and cause membrane fusion (13, 14). There are multiple ubiquitously expressed isoforms of the three SNAREs involved in synaptic vesicle exocytosis, a finding that has led to the hypothesis that SNAREs might be determinants of the specificity of different fusion events within a cell (15). Syt I specifically interacts with the target SNAREs (t-SNAREs) syntaxin 1 and SNAP-25 (16), and these interactions are thought to be key for its proposed function as a Ca2+ sensor for rapid synaptic vesicle exocytosis (10, 17).

In this study, we sought to identify SNARE proteins involved in the Ca2+-triggered fusion of lysosomes with the plasma membrane. In analogy to previous findings on Syt I-regulated synaptic vesicle exocytosis, we hypothesized that the SNARE complex mediating lysosomal exocytosis interacts with Syt VII and consists of a VAMP isoform on the lysosome and syntaxin and/or SNAP-25 isoforms on the plasma membrane. Consistent with this view, our studies identified interactions between Syt VII and a Ca2+-triggered SNARE complex containing the lysosomal vesicle SNARE (v-SNARE) TI (toxin-insensitive)-VAMP/VAMP7 (18, 19) and the t-SNAREs SNAP-23 (20) and syntaxin 4 (21). Lysosomal exocytosis was specifically inhibited by interfering with the capacity of these SNARE proteins to assemble into a complex, thus confirming their functional role in the process.

EXPERIMENTAL PROCEDURES

Antibodies and DNA Constructs—Anti-rat SNAP-23 was purchased from Affinity Bioreagents, Inc. (catalog number PA1–738). Anti-synaptotagmin 2 and 6 antibodies were purchased from Calbiochem (catalog numbers 574786 and 574790), and anti-syntaxin 2 and 4 were purchased from Alomone Labs (catalog numbers ANR-005 and ANR-004). G. Reed (Harvard School of Public Health) kindly provided affinity-
purified anti-syntaxine 4 rabbit antibodies. The anti-Ti-VAMP/VAMP7 monoclonal antibodies (Cl 158.2) were generated in T. Galli's laboratory (22). LY1C6 anti-Lamp-1 monoclonal antibodies were provided by I. Mellman, Yale University. Anti-Syt VII rabbit antibodies were generated as described previously (2). Secondary Alexa Fluor488-labeled anti-mouse IgG was purchased from Molecular Probes, and secondary horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG antibodies from Regeneron & Perry Laboratories, NY.  

To obtain the H3 SNARE domain of syntaxin isoforms, we used the following oligonucleotide primers: syntaxin 2, 5'-CATATCCAGATTAC-TAGGCAAGC-3' (forward) and 5'-GGATCTTTATTTCACTTGCGTGC-TGC-3' (reverse); syntaxin 3, 5'-CATATCAGATTTTGCCAGAAGGCTCCTCAACG-3' (forward) and 5'-GGATCTCTATTCTCTTGCGTGC-TGC-3' (reverse); syntaxin 6, 5'-CATATGCAACGAGCAAGACGACGCGCGGAC-3' (forward) and 5'-GGATCTTTATTTCACTTGCGTGC-TGC-3' (reverse). To obtain the coiled coil domain of VAMP4, we used the primers 5'-CATATGGGACCTAGAATGATATAACG-3' (forward) and 5'-GGATCTTTATTTCACTTGCGTGC-TGC-3' (reverse). To obtain the SNARE domain of Ti-VAMP/VAMP7, we used the primers 5'-CATATGCAACGAGCAAGACGACGCGCGGAC-3' (forward) and 5'-GGATCTCTATTCTCTTGCGTGC-TGC-3' (reverse). These primers all contain an NdeI site in the forward primer and a BamHI site in the reverse primer (underlined). cDNA synthesized from total NRK cell RNA was cleaved by restriction digest, cloned into pET15b (Novagen), and finally incubated in 5 mM free Mg2+ domain of Syt I or Syt VII was bound to Affi-Gel 10 beads in Buffer A for 1 h at 4 °C. The beads were washed three times, and bound proteins were eluted and combined with 3X Sample Buffer and analyzed by SDS-PAGE followed by immunoblotting.  

For binding assays to His-tagged C2A constructs, the His-tagged C2A domain of Syt I or Syt VII was bound to Affi-Gel 10 beads in Buffer A for 1 h at 4 °C. The beads were washed three times, and bound proteins were eluted and combined with 3X Sample Buffer and analyzed by SDS-PAGE followed by immunoblotting.  

Binding Assays—NRK cells were lysed in Buffer A (10 mM HEPES, NaOH, pH 7.4, 0.1 mM NaCl, 1% Nonidet P-40, 2.5 mM MgCl2, 1 mM CaCl2), spun in a microcentrifuge at 14,000 rpm for 5 min, and pre-cleared by incubating with glutathione-Sepharose 4B for 2 h at 4 °C. The supernatant was incubated overnight with glutathione-Sepharose 4B bound to either GST only or GST fused to the C2A domain of Syt VII. The beads were washed three times with Buffer A and eluted with 0.5 mM dithiothreitol in Buffer A, followed by an elution in the same buffer containing 5 mM EGTA instead of 1 mM CaCl2. The eluted fractions were precipitated with trichloroacetic acid, resuspended in 1X Sample Buffer, and analyzed by SDS-PAGE.  

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For toxin cleavage—Recombinant His-tagged BoNT/E was activated by treatment with 20 mM dithiothreitol for 30 min at 37 °C and incubated with NRK lysates prepared as described above for at least 1 h at 37 °C. Reactions were stopped by adding 3X SDS Sample Buffer and boiling for 5 min.  

Toxin Cleavage—Recombinant His-tagged BoNT/E was activated by treatment with 20 mM dithiothreitol for 30 min at 37 °C and incubated with NRK lysates prepared as described above for at least 1 h at 37 °C. Reactions were stopped by adding 3X SDS Sample Buffer and boiling for 5 min. Streptolysin-O Permeabilization and β-Hexosaminidase Secretion—NRK cells were plated in 60-mm dishes at a concentration of 5 × 104 per 4 ml of Dulbecco's modified Eagle's medium + 10% FBS and allowed to grow overnight. Cells were washed in ice-cold β-hexosaminidase Assay Buffer A (20 mM HEPES, 110 mM NaCl, 5.4 mM KCl, 0.9 mM NaHPO4, 10 mM MgCl2, 2 mM CaCl2, and 11 mM glucose) and treated with streptolysin-O (SLO) obtained from Streptococcus pyogenes, at a concentration of 1 µg/ml for 7 min on ice. The cells were then washed twice with ice-cold Low Ionic Strength Medium (LISM, 5 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, 5 mM NaHCO3, 0.5 mM MgCl2, 20 mM HEPES/NaOH, pH 7.4, 10 mM glucose, 220 mM sucrose, 0.5% bovine serum albumin), incubated in LISM at 37 °C for 5 min in the presence of varying concentrations of recombinant protein, and finally incubated in 5 mM free Mg2+ and 2 mM ATP-containing Buffer B (20 mM HEPES, 40 mM KCl, 100 mM potassium glutamate, and 5 mM EGTA) with or without 1 µM free Ca2+ for 10 min at 37 °C. The desired concentrations of free Mg2+ and Ca2+ were obtained with a Ca2+ or Mg2+/EGTA buffering system calculated using the software by Foehr and Hollenberg. Supernatants were collected, and the cells were lysed in Nonidet P-40 to determine the total amount of β-hexosaminidase remaining in the cells. For detection of β-hexosaminidase activity, 350 µl was incubated for 15 min at 37 °C with 50 µl of 6 mM 4-methylumbelliferonyl-N-acetyl-p-glucosaminide in sodium citrate phosphate buffer, pH 4.5. The reaction was stopped by the addition of 100 µl of 2 mM Na2CO3, 1.1 mM glycine, and the fluorescence was measured by excitation at 365 nm, emission 450 nm. For toxin treatment, cells were incubated in LISM at 37 °C for 10 min in the presence of varying concentrations of activated toxin.  

Electroporation, Surface Staining for Lamp-1, and Flow Cytometry—Confluent monolayers of NRK cells were trypsinized and resuspended in balanced salt solution containing 3% FBS, 1% protease 3% FBS, 1% glucose, 1% penicillin/streptomycin, and Na2CO3, NaOH, pH 7, at a concentration of 1 × 106/ml. 400 µl of this suspension was added to a Gene Pulser Cuvette (Bio-Rad), and cells were electroporated at 300 V and 450 microfarads, followed by a 5-min incubation on ice. After 1 min at 37 °C, the cells were returned to ice and stained for surface-exposed Lamp-1. Briefly, cells were resuspended in 50 µl of ice-cold LISM at 4 °C and incubated at 4 °C for 30 min. The cells were then washed, fixed with 2% paraformaldehyde, quenched with 10 mM NH4Cl, and stained with Alexa Fluor488 goat-anti-mouse antibodies (Molecular Probes) for 30 min, resuspended in 0.5 ml PBS, and analyzed by flow cytometry. A fluorescence-activated cell sorter (FACS), FACS-Calibur (BD Biosciences), was used to excite the cells at 488 nm (488 nm), and the emission was collected through a 530/30-nm bandpass filter. A minimum of 10,000 cells was analyzed in each sample. Data analysis was performed using CellQuest (BD Biosciences).  

Microinjection and Surface Staining for Lamp-1—Microinjection and staining for surface Lamp-1 was carried out as described previously (4).
2,5-diphenyltetrazolium bromide (Sigma) was added to each well, and HCl solution was added to each well, and the plate was incubated to recover at 37 °C. Modified Eagle Dulbecco HEPES) containing Texas Red dextran (4). The cells were returned to /H11003

BoNT/E was microinjected into the cells at a concentration of 400 nM in the presence of buffer alone, 1 C, and staining on ice for surface-exposed Lamp-1. Previously activated and/or heat-killed B. burgdorferi was microinjected into the cells at a concentration of 600 nM in microinjection buffer (150 mM potassium gluconate, 2 mM MgCl2, 10 mM HEPES) containing Texas Red dextran (4). The cells were returned to Dulbecco's modified Eagle's medium containing 10% FBS and allowed to recover at 37 °C for 2 h, followed by treatment with 10 μM ionomycin for 5 min at 37 °C, and staining on ice for surface-exposed Lamp-1.

**RESULTS**

The v-SNARE TI-VAMP/VAMP7 Co-localizes with Syt VII on Lysosomes—The v-SNARE TI-VAMP/VAMP7 (18) was recently shown to be specifically targeted to late endosomes and lysosomes of HeLa cells by its amino-terminal Longin domain (19). This finding is consistent with prior observations in NRK cells, in which Myc-tagged TI-VAMP/VAMP7 was targeted to compartments containing the lysosomal glycoprotein Lamp-1 (25). Immunofluorescence with a monoclonal antibody against TI-VAMP/VAMP7 (22) confirmed that endogenous TI-VAMP/VAMP7 is present on lysosomes in NRK cells. Extensive overlap of TI-VAMP/VAMP7 with Lamp-1 (Fig. 1A) and Syt VII (Fig. 1B) staining was observed. Previous studies showed that in NRK cells Syt VII is localized on Lamp-1-positive, dense lysosomes, which also contain the processed form of cathepsin L (2). Therefore, these data identified TI-VAMP/VAMP7 as a candidate for involvement in a complex with plasma membrane t-SNAREs to drive lysosomal exocytosis, because it is the only v-SNARE detected so far on the membrane of mature lysosomes in mammalian cells. Consistent with this view, endogenous TI-VAMP/VAMP7 was detected on recently formed intracellular vacuoles containing T. cruzi, the protozoan parasite that utilizes Ca2+-regulated lysosomal exocytosis for invasion (Fig. 1C) (6).

**TI-VAMP/VAMP7 Interacts with the Plasma Membrane t-SNAREs Syntaxin 4 and SNAP-23**—Previous studies indicated that syntaxins 2–4 and SNAP-23 are ubiquitously expressed plasma membrane t-SNAREs (21). SNAP-23, a ubiquitously expressed isoform of SNAP-25, has been detected on the plasma membrane of many cell types (26). It was initially cloned as a syntaxin 4-interacting protein, although it was also shown to be capable of binding multiple syntaxin isoforms in vitro, as well as VAMP1 and VAMP2 (20). To determine whether SNAP-23 and the known plasma membrane syntaxins were expressed in NRK cells, we prepared a purified plasma membrane fraction. Monolayers of NRK cells were surface-biotynlated, and disrupted cells were incubated with avidin-coated beads to specifically pull down the plasma membrane. Western blot with isoform-specific antibodies detected SNAP-23, syntaxin 3, and syntaxin 4 on the plasma membrane fraction of NRK cells, whereas TI-VAMP/VAMP7 and syntaxin 2 were absent (Fig. 2A). Thus, syntaxin 3 and 4 and SNAP-23 represented potential t-SNARE partners for TI-VAMP/VAMP7 in lysosomal exocytosis.

To determine which t-SNAREs were the best candidates for this role, co-immunoprecipitation experiments were performed in NRK cell lysates. As shown in Fig. 2B, a monoclonal antibody specific for TI-VAMP/VAMP7 co-immunoprecipitated syntaxin 4, but not syntaxin 2 or 3 or SNAP-23 from total NRK cell lysates. These findings are consistent with recent experiments in HeLa cells, which detected SNARE complexes containing green fluorescent protein-tagged TI-VAMP/VAMP7 and syntaxin 4 (19). In pulldown experiments, we found that endogenous syntaxin 2 and syntaxin 4 bound to GST-SNAP-23 but not to GST alone (Fig. 2C). This interaction seemed to be specific for these isoforms, as syntaxin 3 and syntaxin 6 (a Golgi syntaxin (27)) did not bind to GST-SNAP-23. Co-immunoprecipitation experiments, however, detected a preferential interaction of endogenous SNAP-23 with syntaxin 4 and not syntaxins 2 and 3 (Fig. 2D).

**Syt VII Interacts with the t-SNAREs Syntaxin 4 and SNAP-23**—SNAP-23 has been shown to be involved in several Ca2+-regulated exocytic events, including neutrophil degranulation (28), secretion of dense core granules in platelets (29), mast cell degranulation (30), and zymogen granule exocytosis in pancreatic acinar cells (31). In neuroendocrine cells, SNAP-23 was recently shown to interact with Syt VII C2A-B in a Ca2+-dependent manner (32). The C2A domain of Syt VII has the capacity to bind SNAP-25-syntaxin 1a heterodimers with high affinity (33), a property not exhibited by the Syt I C2A domain (33, 34). Consistent with these results, we found that the Syt VII C2A domain (previously shown to inhibit lysosomal exocytosis (2)) and not the Syt I C2A domain interacts with endogenous SNAP-23 in NRK cells (results not shown) and with GST-SNAP-23 in vitro (Fig. 3, A and B). In the presence of 1 mM Ca2+, GST-SNAP-23 specifically associated with His-tagged Syt VII C2A, but not Syt I C2A (Fig. 3A). Most interesting, the interaction of GST-SNAP-23 with His-tagged Syt VII C2A was enhanced in the presence of the I3 domain (the region involved in the formation of SNARE coiled-coil bundles) of syntaxin 4 but not of syntaxins 2, 3, or 6 (Fig. 3B).
We next examined the material pulled down from NEM-treated lysates (to inactivate N-ethylmaleimide-sensitive factor and stabilize SNARE complexes (18)) by beads coated with His-tagged C2A domains of Syt VII or of Syt I, for the presence of plasma membrane syntaxins which could be part of a SNARE complex with SNAP-23. It was reported previously (8) that the Syt VII C2A domain interacts in a Ca\(^{2+}\)-dependent manner with multiple syntaxin isoforms in vitro. Our results confirmed this observation, but we also found that Syt VII C2A bound syntaxin 4 more effectively than Syt I C2A, whereas syntaxin 2 and 3 seemed to bind both synaptotagmin isoforms equally well (Fig. 3C). Our data thus suggests that syntaxin 4 and SNAP-23 are good candidates for t-SNAREs involved in a SNARE complex mediating lysosomal exocytosis, as both are located on the plasma membrane of NRK cells and specifically interact with Syt VII under conditions that preserve SNARE complexes.

**Ca\(^{2+}\) Triggers Formation of an SDS-resistant SNARE Complex Containing TI-VAMP/VAMP7, Syntaxin 4, and SNAP-23**—One characteristic of SNAREs is their ability to form high molecular weight SDS-resistant complexes that can only be dissociated upon boiling (35). In order to determine whether specific SDS-resistant SNARE complexes were formed upon lysosome exocytosis, we wounded NRK cells by scraping from the dish. This procedure causes plasma membrane wounding and Ca\(^{2+}\) influx in the majority of the cell population, triggering lysosomal exocytosis and rapid resealing (4). NEM-treated NRK cells scraped in the presence of Ca\(^{2+}\) were solubilized directly in SDS sample buffer, a condition that does not allow de novo formation of SNARE complexes in solution (35). Half of
each sample was boiled, and the other half was kept at room temperature. Upon SDS-PAGE followed by Western blot, we detected high molecular weight, heat-sensitive complexes recognized by antibodies to syntaxin 4, SNAP-23, and TI-VAMP/VAMP7 (Fig. 4, arrowheads). These bands, which migrated with an apparent molecular mass greater than 220 kDa, were only present when cells were scraped in the presence of 1 mM Ca\(^{2+}\)/H\(_{11001}\). SDS-resistant SNARE complexes containing endogenous SNAP-25, syntaxin, and synaptobrevin/VAMP2 have been reported to form a ladder of bands varying in size from 60 to 300 kDa in cell lysates (36). Under our conditions, which do not involve massive exocytosis as observed in specialized secretory cells (only about 10% of the total lysosomal population fuses with the plasma membrane upon Ca\(^{2+}\) influx (1)), SDS-resistant complexes of greater than 220 kDa in size were the most readily detectable. Thus, the presence of TI-VAMP/VAMP7, SNAP-23, and syntaxin 4 in a Ca\(^{2+}\)-dependent, SDS-resistant complex is consistent with the participation of these SNAREs in a complex responsible for lysosomal exocytosis.

We predict that Syt VII regulates the formation of SNARE complexes promoting lysosomal exocytosis. Therefore, we also examined whether the SNARE proteins found to interact with Syt VII also bound TI-VAMP/VAMP7. As described above, His-tagged Syt I or Syt VII C\(_{2A}\) domains were used in pulldown experiments from NEM-treated NRK lysates. Western blotting detected TI-VAMP/VAMP7 bound to the C\(_{2A}\) domain of Syt VII and not to the equivalent domain of the brain isoform Syt I (Fig. 4A). In a similar experiment, a GST fusion of the Syt VII C2A domain was used in pulldown experiments from NEM-treated NRK lysates. Proteins were eluted from the beads using high salt, followed by an elution with EGTA. Western blotting confirmed that TI-VAMP/VAMP7 bound to GST-Syt VII C2A and not to GST alone, and also showed that more TI-VAMP/VAMP7 was recovered in the EGTA elution, indicating a Ca\(^{2+}\)-dependent interaction with Syt VII (Fig. 4C). These data suggest that TI-VAMP/VAMP7 interacts in a Ca\(^{2+}\)-dependent manner with Syt VII, possibly as part of a SNARE complex.
Functional Role of TI-VAMP/VAMP7, Syntaxin 4, and SNAP-23 in Lysosomal Exocytosis—A major piece of evidence supporting a role for SNAREs in controlling specific membrane fusion events is the inhibitory effect of recombinant α-helical coil domains, the regions involved in the formation of SNARE complexes. In cracked PC12 cells, the SNARE domains of VAMP2 and syntaxin 1a are more effective in inhibiting norepinephrine secretion, when compared with other isoforms (15). We showed previously that the recombinant Syt VII C2Ad o-domain specifically inhibits Ca\(^{2+}\)-dependent lysosomal exocytosis using a β-hexosaminidase secretion assay in SLO-permeabilized NRK cells (2). We examined the effect of adding His-tagged SNARE domains of VAMP4 or TI-VAMP/VAMP7, or syntaxin 4 or 6, or of VAMP4 (37) or syntaxin 6 (27) as controls, to this assay. A dose-dependent inhibition of β-hexosaminidase release was observed in the presence of the H3 domain of syntaxin 4, reaching an inhibition of about 37% at 15 μM (Fig. 5B). The same domain from the Golgi isoform, syntaxin 6, did not inhibit exocytosis at any concentrations tested. Similar dose-dependent inhibition was seen with the SNARE domain of TI-VAMP/VAMP7, reaching an inhibition of about 35% at 12.5 μM (Fig. 5A). The SNARE domain of VAMP4, a Golgi VAMP isoform, had no effect. In previous studies (15) using cracked PC12 cells, similarly high concentrations of soluble SNARE domains were required to achieve inhibition of exocytosis.

Previous work from our laboratory showed that lysosomal exocytosis has an important role in the resealing of plasma membrane wounds (4). We thus proceeded to verify whether specific recombinant SNARE domains were also inhibitory in a more physiological assay of plasma membrane wounding and repair. Cells were wounded by electroporation, and the rapid exocytosis-mediated repair was measured by detecting surface exposure of the luminal epitope of Lamp-1, measured by FACS.
with the equivalent domain from VAMP4 (Fig. 5D). The results of these two independent assays, which measured β-hexosaminidase release in SLO-permeabilized cells or the surface exposure of Lamp-1 after resealing of wounded cells, indicate that syntaxin 4 and VAMP7 are functionally involved in Ca²⁺-triggered lysosomal exocytosis.

SNAREs were first identified as candidates for involvement in membrane fusion due to their specific cleavage by clostridial neurotoxins (38, 39). Consequently, these neurotoxins have been widely used as tools to dissect the function of specific SNARE proteins. Although botulinum neurotoxin serotype E (BoNT/E) specifically cleaves SNAP-25 at its COOH terminus, human and mouse SNAP-23 were found to be resistant to cleavage (18, 40). However, BoNT/E was subsequently shown to be able to cleave canine and rat SNAP-23, also at a COOH-terminal site (24, 41). To confirm that effective cleavage of SNAP-23 could be achieved in NRK (rat) cells, we monitored the activity of purified His-tagged recombinant BoNT/E on cell extracts by using Western blot. Our antibody recognizes the extreme COOH terminus of SNAP-23, so cleavage is indicated by the disappearance of the SNAP-23 immunoreactive band. Near complete cleavage was observed after 1 h, at concentrations between 10 and 100 nM (Fig. 6A).

We proceeded to examine whether SNAP-23 cleavage by BoNT/E had an effect on lysosomal exocytosis. Cells were permeabilized with SLO, and the toxin was added for 10 min at 37 °C, and 1 μM Ca²⁺ was added to trigger exocytosis. Secretion of the lysosomal enzyme β-hexosaminidase was inhibited in a dose-dependent manner, with an inhibition of 51% reached at 4.4 μM BoNT/E. In contrast, no inhibition was observed when the cells were incubated with the same concentrations of heat-inactivated toxin (Fig. 6B). Given that only partial cleavage was probably achieved in the short 10-min incubation period allowed by the SLO permeabilization assay, these results strongly suggested that SNAP-23 is essential for lysosomal exocytosis. For this reason we also microinjected the toxin directly into NRK cells, and we looked for an effect on lysosomal exocytosis after a longer incubation period. BoNT/E was microinjected at a concentration of 400 nM, along with Texas Red dextran, to allow for subsequent identification of injected cells. After a 2-h incubation at 37 °C to allow full cleavage, the cells were stimulated to undergo lysosomal exocytosis by the addition of 10 μM ionomycin for 3 min (1). Exocytosis was monitored by exposure of the luminal epitope of Lamp-1, which we detected through immunofluorescent surface staining of non-permeabilized cells (1, 2). All cells that were microinjected with the heat-inactivated toxin showed Lamp-1 surface staining following ionophore treatment. In contrast, injection of the active toxin caused a marked inhibition in Lamp-1 surface exposure, when compared with neighboring uninjected cells (Fig. 6C). In addition, the inhibitory effect of SNAP-23 cleavage on lysosomal exocytosis was shown not to be due to a generalized decrease in cell viability. NRK cells scraped from the dish in the presence of 1 μM toxin fully recovered and showed normal viability after 4 h in an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). These results thus confirm that the t-SNARE SNAP-23 plays an essential role in Ca²⁺-triggered exocytosis of lysosomes, probably by participating in the formation of a SNARE complex containing syntaxin 4 and TI-VAMP/VAMP7.

**DISCUSSION**

In this study we identified the v-SNARE TI-VAMP/VAMP7 and the t-SNAREs SNAP-23 and syntaxin 4 as components of a SNARE complex involved in the Ca²⁺-triggered exocytosis of conventional lysosomes. Several independent lines of evidence support this conclusion. First, TI-VAMP/VAMP7 is localized on lysosomes, and in cell lysates it interacts specifically with the plasma membrane t-SNAREs SNAP-23 and syntaxin 4, as well as with the lysosomal exocytosis regulatory protein Syt VII.
Second, interaction of SNAP-23 with the C2A domain of Syt VII is \(\text{Ca}^{2+}\)-dependent and facilitated by the SNAPRE domain of syntaxin 4. Third, \(\text{Ca}^{2+}\) triggers formation of a membrane-associated, high molecular weight SDS-resistant complex containing TI-VAMP/VAMP7, SNAP-23, and syntaxin 4. Fourth, SNAPRE domains of TI-VAMP/VAMP7 and syntaxin 4, or cleavage of SNAP-23 by BoNT/E, inhibit \(\text{Ca}^{2+}\)-triggered lysosomal exocytosis in NRK cells.

The SNAPRE complex formed by syntaxin 1, SNAP-25, and VAMP2 during synaptic vesicle exocytosis resists SDS dissociation due to the formation of a highly stable coiled-coil bundle, which consists of four \(\alpha\)-helical domains contributed by the three members (35, 36). Work with recombinant proteins showed that many different SNAPRE proteins can form SDS-resistant complexes in vitro (42, 43), but their ability to associate promiscuously in vivo is significantly more restricted, probably due to the presence of accessory proteins that promote correct pairing (15, 44). Thus, although SDS-resistant complexes can be formed \textit{de novo} after cells are solubilized in Triton X-100, co-immunoprecipitation experiments have proven to be a reliable approach in the identification of components of functional SNAPRE complexes. In our study, regardless of the antibody used for immunoprecipitation, this approach consistently identified syntaxin 4, and not syntaxin 2 or 3, as a plasma membrane component of a complex also containing SNAP-23 and TI-VAMP/VAMP7.

Syntaxin 4 has been implicated in other regulated exocytic trafficking pathways, including the translocation of GLUT4 in the plasma membrane of rat adipose cells. In that study, it was proposed to bind SNAP-23 and VAMP2 and/or VAMP3, forming a specific SNAPRE complex (45). In RBL-2H3 mast cells, syntaxin 4 was shown to be functionally involved in the exocytosis of secretory granules and to bind SNAP-23 and different VAMP isoforms although not to bind TI-VAMP/VAMP7 (46). In these cells, however, TI-VAMP/VAMP7 was shown to translocate to the plasma membrane upon stimulation (47). In platelets, SNAP-23 as well as syntaxins 2 and 4 have been implicated in \(\alpha\)-granule release (48, 49). Syntaxin 4 is therefore emerging as a multifunctional t-SNARE on the plasma membrane, which along with SNAP-23 appears to be able to mediate the exocytosis of various compartments, possibly by forming complexes with distinct v-SNAREs.

After triggering lysosomal exocytosis by scrape-wounding NRK cells, we detected formation of a high molecular weight SDS-resistant complex of greater than 220 kDa, recognized by antibodies to VAMP7, SNAP-23, and syntaxin 4 (Fig. 4A). This complex was \(\text{Ca}^{2+}\)-dependent and dissociated by boiling, leading us to conclude that it was likely to correspond to a trans-SNARE complex between the lysosome and the plasma membrane containing TI-VAMP/VAMP7, SNAP-23, and syntaxin 4. In these experiments, however, we also detected a \(\text{Ca}^{2+}\)-independent complex that was slightly smaller, containing SNAP-23 and syntaxin 4, but not TI-VAMP/VAMP7. This complex may reflect SNARE complexes involved in constitutive traffic of other vesicles to the plasma membrane, possibly involving a distinct VAMP isoform. Alternatively, these \(\text{Ca}^{2+}\)-independent complexes may consist of SDS-resistant hetero-oligomers of SNAP-23 and syntaxin 4. Multiple SDS-resistant complexes have been detected in chromaffin cells, and the majority of these complexes contained only SNAP-23 and syntaxin 1 as a stable dimer on the pathway to the ternary SNAPRE complex (50), with only a small subset containing VAMP2 (51). But it is important to note that a heterogeneous band pattern is also routinely observed with SDS-resistant ternary SNAPRE complexes from whole cell lysates (36), a phenomenon that has also been attributed to different folding intermediates, as opposed to multimers of complexes (51).

Earlier studies showed that the soluble C2A domain of Syt VII, or affinity-purified antibodies against this domain, has a strong inhibitory effect on the \(\text{Ca}^{2+}\)-triggered exocytosis of lysosomes, as well as plasma membrane resealing and host cell invasion by \textit{T. cruzi} (2, 4, 7). In addition to a \(\text{Ca}^{2+}\)-dependent Syt VII-SNARE-23 interaction, consistent with what was previously described in neuroendocrine cells (32), we also detected TI-VAMP/VAMP7 in pulldown assays with the Syt VII C2A domain but not with the Syt I C2A domain (Fig. 4B and C). Syt I is known to bind to the heterotrimeric complex consisting of syntaxin 1, SNAP-23, and VAMP2 (52, 53), and there is no strong evidence indicating a direct interaction between the C2A domain of Syt I and VAMP2. Our data suggests that the interaction we detected was also between Syt VII C2A and a SNAPRE complex containing TI-VAMP/VAMP7, in addition to SNAP-23 and syntaxin 4 (Fig. 4A).

Previous studies indicated that lysosomal exocytosis plays an important role in the mechanism used by the human parasite \textit{T. cruzi} to invade host cells and in the repair of plasma membrane wounds (4, 7). A role for the lysosomal SNAPRE TI-VAMP/VAMP7 in the exocytic events mediating repair is consistent with the deficient resealing observed in Madin-Darby canine kidney cells expressing the dominant-negative amino-terminal \textit{Longin} domain (54) of TI-VAMP/VAMP7 (results not shown). Identification of specific SNAPRE proteins involved in this process opens the possibility for specific intervention strategies in trypanosome infections. Our findings also represent, to our knowledge, the first description of synaptotagmin-SNARE protein interactions regulating \(\text{Ca}^{2+}\)-triggered exocytosis in non-specialized secretory cells. Our findings suggest that additional members of the synaptotagmin family may also function by regulating the formation of SNAPRE complexes involved in specific \(\text{Ca}^{2+}\)-dependent membrane fusion events.

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Identification of SNAREs Involved in Synaptotagmin VII-regulated Lysosomal Exocytosis
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