SNAP-23 Functions in Docking/Fusion of Granules at Low Ca^{2+}

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Ca^{2+} -triggered exocytosis of secretory granules mediates the release of hormones from endocrine cells and neurons. The plasma membrane protein synaptosome-associated protein of 25 kDa (SNAP-25) is thought to be a key component of the membrane fusion apparatus that mediates exocytosis in neurons. Recently, homologues of SNAP-25 have been identified, including SNAP-23, which is expressed in many tissues, albeit at different levels. At present, little is known concerning functional differences among members of this family of proteins. Using an *in vitro* assay, we show here that SNAP-25 and SNAP-23 mediate the docking of secretory granules with the plasma membrane at high (1 μM) and low (100 nM) Ca^{2+} levels, respectively, by interacting with different members of the synaptotagmin family. In intact endocrine cells, expression of exogenous SNAP-23 leads to high levels of hormone secretion under basal conditions. Thus, the relative expression levels of SNAP-25 and SNAP-23 might control the mode (regulated vs. basal) of granule release by forming docking complexes at different Ca^{2+} thresholds.

INTRODUCTION

In endocrine cells and neurons, an increase in intracellular Ca^{2+} induces release of granules and synaptic vesicles (Rettig and Neher, 2002). Involved in the process is the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, which is composed of syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) that reside in the plasma membrane and synaptobrevin (or vesicle-associated membrane protein-2 [VAMP-2]) in the vesicle membrane (Sollner *et al.*, 1993). In support of the concept that SNAREs are involved in membrane fusion, it has been shown that specific clostridial neurotoxins that cleave the SNARE complex components are also potent inhibitors of neurotransmitter and hormone release in neurons and endocrine cells, respectively (Montecucco, 1998; Jahn and Südhof, 1999). Studies in permeabilized PC12 cells indicated that membrane fusion is triggered by Ca^{2+} -dependent SNARE complex formation (Chen *et al.*, 1999). However, an increase in intracellular $[\text{Ca}^{2+}]$ seems to act at multiple steps in the regulated exocytotic pathway both by inducing the immediate release of vesicles (burst) and the recruitment of new vesicles into the releasable pool (sustained component of release) (Rettig and Neher, 2002). By using antibodies against SNAP-25 in permeabilized chromaffin cells it has been proposed that SNARE complex assembly is involved at both of these steps (Xu *et al.*, 1999). The recruitment of

vesicles into the releasable pool is dependent both on Ca^{2+} and ATP (Bittner and Holz, 1992) and is proposed to involve a population of granules already positioned in the vicinity of the plasma membrane (Neher, 1998; Olofsson *et al.*, 2002).

To study the effects of Ca^{2+} at steps that precede vesicle fusion, we have recently established a functional docking assay that measures binding of granules to the plasma membrane (Chieriegatti *et al.*, 2002). By using this assay, we have found that SNAP-25 interacts in a Ca^{2+} -dependent manner with the granule component synaptotagmin 1 (Syt1) to support an initial and reversible interaction between the vesicle and the plasma membrane (Ca^{2+} -dependent docking). This step precedes another ATP-dependent event that makes association of the granule with the plasma membrane irreversible. These results suggest that the Ca^{2+} -dependent SNAP-25–Syt1 interaction is important at a step of functional granule docking to the plasma membrane that precedes ATP-dependent maturation (priming) (Klenchin and Martin, 2000), formation of the SNARE complex, and fusion. In addition, it has been proposed that direct binding of SNAP-25 and Syt1 is necessary for Ca^{2+} -dependent triggering of membrane fusion and neurotransmitter release (Gerona *et al.*, 2000; Zhang *et al.*, 2002; Earles *et al.*, 2001). In this view, Syt1 operates as the Ca^{2+} sensor that triggers opening of the fusion pore (Littleton *et al.*, 1999; Wang *et al.*, 2001). Moreover, genetic and biochemical evidence supports the concept that Syt1 is involved in endocytosis (reviewed by Chapman, 2002; Slepnev and De Camilli, 2000; Südhof, 2001). Thus, the emerging view is that synaptotagmins may function at multiple steps during the trafficking of secretory vesicles. Whereas the function of synaptotagmins as Ca^{2+} sensors in fusion-pore dynamics and vesicle recycling is more established, less is known about their proposed role in vesicle docking to the plasma membrane (Reist *et al.*, 1998; Chieriegatti *et al.*, 2002).

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Abbreviations used: BoNT/C, botulinum toxin C; BoNT/E, botulinum toxin E; SNAP-25, synaptosome-associated protein of 25 kDa; Syt, synaptotagmin; TeNT, tetanus toxin; VAMP, vesicle-associated membrane protein.

SNAP-25 is specifically expressed in neurons and in endocrine cells (Oyler *et al.*, 1989; Sadoul *et al.*, 1995). Differently, the SNAP-25 homologue SNAP-23 (also referred to as Syndet), is expressed at relatively low levels in endocrine cells and neurons compared with many other types of cells. Like SNAP-25, SNAP-23 is localized at the plasma membrane (Ravichandran *et al.*, 1996; Wang *et al.*, 1997). SNAP-23 has been shown to play a role in the translocation to and fusion with the plasmalemma of a specialized type of exocytotic vesicle, the Glut4 vesicle (Rea *et al.*, 1998). The latter process is regulated by insulin and can occur at resting intracellular calcium concentration ($[Ca^{2+}]_i$) (Whitehead *et al.*, 2001). Moreover, constitutive docking and fusion of vesicles at the plasma membrane must occur in every cell at resting $[Ca^{2+}]_i$ levels to carry newly synthesized lipids and protein to the plasma membrane. Finally, Ca^{2+} -dependent recruitment and release of vesicles seems to occur in many, perhaps in all cell types (Dan and Poo, 1992; Chavez *et al.*, 1996; Borgonovo *et al.*, 2002). These observations are consistent with the idea SNAP-25 functions in vesicle release at elevated Ca^{2+} , whereas SNAP-23 can, at least in some cells, function in exocytosis at resting Ca^{2+} levels.

In our previous work, we have shown that SNAP-25-dependent granule docking to plasma membranes occurs at high Ca^{2+} (1 μ M) and requires the interaction of SNAP-25 with Syt1. It has been recently proposed that specific synaptotagmins bind to Ca^{2+} with ~10-fold higher apparent affinity than Syt1 (Shin *et al.*, 2002; Sugita *et al.*, 2002). On the basis of these considerations, we hypothesized that SNAP-23 supports vesicle docking at resting $[Ca^{2+}]_i$ levels by interacting with synaptotagmins that bind Ca^{2+} with high affinity. Here, by using the in vitro docking assay and a cell-based secretion assay, we show that SNAP-25 and SNAP-23 function in granule docking and fusion at elevated and low Ca^{2+} , respectively, by interacting with different members of the synaptotagmin family.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibody (mAb) against SNAP-25 (SMI 81) was from Sternberg Monoclonals (Lutherville, MD); mouse mAb against the $\alpha 1$ subunit of Na^+/K^+ ATPase was from Upstate Biotechnology (Lake Placid, NY); mouse mAb 41.1 against Synaptotagmin 1 was from Synaptic Systems (Göttingen, Germany); mouse monoclonal antibodies against β -Gal and green fluorescent protein (GFP) were from Roche Diagnostics (Indianapolis, IN); rabbit polyclonal antibody against Calnexin was from Stressgen Biotechnologies (Victoria, BC, Canada); mouse polyclonal antibody against Myc was from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal antibody against SNAP-23 has been described previously (Wang *et al.*, 1997); rabbit polyclonal antibody against GFP was from BD Biosciences Clontech (Palo Alto, CA); mouse mAb against syntaxin 1 (clone HPC-1) was from Sigma-Aldrich (St. Louis, MO) (Inoue *et al.*, 1992); mouse mAb 69.1 against VAMP-2 was from Synaptic Systems.

BoNT and Syt Protein Expression and Purification

The cDNA of the recombinant His₆-tagged light chain of Botulinum Toxin E (BoNT/E) in the vector pBN17 and recombinant His₆-tagged light chain of Botulinum Toxin C (BoNT/C) in the vector pBN27 (Binz *et al.*, 1994) were transformed into *Escherichia coli* strain M15/pRep. The His₆-tagged protein was purified by chromatography by using Ni-NTA agarose (QIAGEN, Valencia, CA) following the manufacturer's instructions. The pGEX-2T plasmids encoding GST-Syt1-12 C2AB, which corresponds to the entire cytoplasmic domain of Syt1-12, and glutathione S-transferase (GST)-Syt1C2A, which corresponds to the C2A domain of Syt1 have been described previously (Desai *et al.*, 2000). The GST-coupled Syts were expressed in JM109 bacterial cells, purified, and analyzed as described previously (Desai *et al.*, 2000). The light chain of Tetanus toxin (TeNT) was a kind gift of Dr. Montecucco (Rossetto *et al.*, 2001).

Cell Fractionation and In Vitro Assay

AtT-20 cells or N2A cells were washed once in Kglu buffer (20 mM HEPES, pH 7.4, 120 mM potassium glutamate, 20 mM potassium acetate, 5 mM EGTA,

1 mg/ml bovine serum albumin) and scraped from plates in the Kglu buffer. Cells were broken by passing them three times through a 27-gauge 1/2 syringe needle and centrifuged at $100 \times g$ for 5 min. The postnuclear supernatant was centrifuged at $7200 \times g$ for 10 min to obtain a plasma membrane-containing fraction PII. The supernatant of this centrifugation corresponds to the cytoplasm and granule-containing fraction SII (Koticha *et al.*, 2002). The pellet PII was incubated for 30 min at 30°C to release additional bound granules (Chieregatti *et al.*, 2002) and re-centrifuged at $7200 \times g$ for 10 min. This pellet corresponds to the P fraction used for the in vitro docking assay (see below).

In vitro docking assays were conducted as described previously (Chieregatti *et al.*, 2002) in 0.12-ml total volume reactions that contained the $7200 \times g$ pellet P derived from either AtT-20 cells or N2A cells resuspended in Kglu buffer with 5 mM EGTA. The granule-containing fraction SII derived from N2A cells transiently expressing the chimera POMC- β -Gal was added to the P fraction and $CaCl_2$ was adjusted to obtain 10 μ M free $[Ca^{2+}]$ to induce docking. The sample was incubated for 10 min at 30°C. At the end of the incubation time, samples were centrifuged again at $7200 \times g$ for 10 min to obtain plasma membranes with or without docked granules. Granules docked to the plasma membrane were analyzed by Western Blot probed with antibodies against β -Gal and Na^+/K^+ ATPase. The intensity of bands corresponding to processed POMC- β -Gal 120/124-kDa peptides stored in granules (Koticha *et al.*, 2002) was measured by densitometry. Western blots analysis was performed by using enhanced chemiluminescence (Wang *et al.*, 1997). When indicated, P membranes were pretreated before the docking reaction for 30 h at 30°C with 500 nM recombinant His₆-tagged light-chain of Botulinum Toxin E (Binz *et al.*, 1994), (Chieregatti *et al.*, 2002). For the inhibition experiments using recombinant Syt proteins, granules and plasma membrane were mixed and preincubated with the Syt proteins (5 μ M) for 10 h at 30°C. As Synaptotagmins bind Ca^{2+} , for these experiments, an excess of free 100 μ M $[Ca^{2+}]$ was added to induce docking. Where indicated, granules docked to the plasma membrane were released by incubation with addition of 20 mM EGTA (from a 0.5 M EGTA stock solution, pH 7.4) for 10 min at 30°C.

To determine whether AtT-20, and N2A cells express Syt7 and Syt3, cells and mouse brain were homogenized in phosphate-buffered saline containing protease inhibitors (Complete Mini; Roche Diagnostics); postnuclear supernatants were obtained after centrifugation of the homogenates at $100 \times g$ for 5 min.

Immunoprecipitations

For the immunoprecipitation reaction, samples with plasma membrane-containing P fraction were incubated with granule-containing SII fraction in the presence or in the absence of 10 μ M free calcium for 10 min at 30°C. After the docking reaction, samples were diluted 1:5 with IP buffer (100 mM Tris, pH 7.4, 50 mM NaCl, 0.5% Triton X-100, and protease inhibitors) with addition of EGTA and $CaCl_2$ to reach a final concentration of 50 μ M free Ca^{2+} in all the samples. The addition of 50 μ M free Ca^{2+} in the IP buffer was done to prevent dissociation of protein complexes that are formed in the docking step (Chieregatti *et al.*, 2002). Immunoprecipitations were done by adding anti-Synaptotagmin antibody 41.1 (p65), or mouse mAb against GFP, or affinity-purified rabbit antibodies against SNAP-23 as indicated. Samples were rotated for 1 h at 4°C. After addition of protein G beads the samples were further incubated for 1 h at 4°C. After five washes with IP buffer at pH 7.4, the beads were resuspended in sample buffer, boiled for 5 min, and centrifuged. Supernatants were loaded onto the SDS-PAGE gel. Western blots were probed with anti-Synaptotagmin antibody 41.1, or with rabbit polyclonal antibodies against GFP.

Cell Culture and Transfection

The plasmids POMC- β -Gal-pcDNA3, SNAP-25A-Myc-pcB7, and *Syndet*(SNAP-23)-pcB7 for exogenous protein expression in mammalian cells have been described previously (Koticha *et al.*, 2002). The plasmids pCMV5-Syt1-EYFP, pCMV5-SytVIII-EYFP, and pCMV5-SytIII-EYFP have been described previously (Sugita *et al.*, 2001, 2002). The cDNAs encoding for the yellow fluorescent protein (YFP)-tagged cytoplasmic domains of Syt1, (Syt1C2AB-EYFP, from amino acid residue 96 of Syt1) and of Syt7 (Syt7C2AB-EYFP, from amino acid residue 93 of Syt7) were amplified by polymerase chain reaction (PCR) from pCMV5-Syt1-EYFP and pCMV5-SytVIII-EYFP by using the forward primers 5'CACCATGGGAGGAAAGAACGCCATTAAACA (the underlined sequence corresponds to Syt1 cDNA) and 5'CACCATGGAGTCTGACCGCAGAACGGA (the underlined sequence corresponds to Syt7 cDNA), and the reverse primer 5'TTACTTGTACAGCTCGTCATGCCGA (the sequence corresponds to EYFP cDNA including the stop codon). The PCR products were subcloned into the pcDNA/GW/p-TOPO vector (Invitrogen, Carlsbad, CA) for mammalian cell expression following the manufacturer's instructions to obtain the pcDNA/GW/p-TOPO-Syt1C2AB-EYFP and pcDNA/GW/p-TOPO-Syt7C2AB-EYFP vectors. The plasmid SNAP-25A-Myc-pEGFP.C1 with GFP at the N terminus of SNAP-25 was obtained by subcloning the SNAP-25A-Myc insert, excised by *SacI* and *XbaI* from the vector SNAP-25A-Myc-pcB7, into the vector pEGFP.C1 digested with *SacI* and *XbaI*. The plasmid SNAP-23-pEGFP.C1 was generated by subcloning SNAP-23 cDNA excised by *HindIII* and

Figure 1. SNAP-25- and SNAP-23-dependent docking. (A) Plasma membrane-containing fraction P from AtT-20 cells (AtT-20) and AtT-20 cells expressing SNAP-23 (AtT-20/SNAP-23, stable transfection; Koticha *et al.*, 1999) were preincubated with or without 500 nM BoNT/E and mixed with granules (fraction SII) derived from N2A transiently transfected with POMC-β-Gal (see MATERIALS AND METHODS). Samples were incubated in the presence or absence of 10 μM free Ca²⁺ to induce docking and further incubated with or without 20 mM EGTA. Granules that co-fractionated with the plasma membrane in the pellet after centrifugation at 7200 × g for 10 h (docked granules) were analyzed by Western blot with antibodies against the plasma membrane marker Na⁺/K⁺ ATPase and β-Galactosidase. (B) Plasma-containing fraction P from AtT-20/SNAP-23 were incubated with 500 nM BoNT/E. Samples were analyzed by Western blot with antibodies against SNAP-25 and SNAP-23. (C) The docking assay was done with plasma membrane fraction P derived from N2A cells and granules derived from N2A transiently transfected with POMC-β-Gal. Samples were incubated

at 4 and 30°C in the presence or absence of 10 μM free Ca²⁺ to induce docking and further incubated in the presence of EGTA as in A. After centrifugation, granules docked to the plasma membrane (pellet, 100% of total sample) and undocked granules in the supernatants (50% of total sample) were analyzed by Western blot as described in A.

*Xba*I from the SNAP-23 (*Syndet*)-pcB7 vector (Koticha *et al.*, 1999) into the vector pEGFP.C1 digested with *Sac*I and *Xba*I.

Mouse neuroblastoma N2A and AtT-20 cells were grown in DMEM with 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, and containing 8 and 10% heat-inactivated fetal calf serum, respectively. HEPES at pH 7.4 (20 mM) was added to the medium of N2A cells. For transient expression, cells were transfected with the DNA of interest by using LipofectAMINE (Invitrogen), according to the manufacturer's instructions. The G14 cell line derived from N2A cells stably expressing BoNT/E light chain and AtT-20 cell line stably expressing SNAP-23 have been described previously (Koticha *et al.*, 1999, 2002). To prepare AtT-20 cell line 6#5 expressing Myc-tagged SNAP-25A (stable transfection), cells were transfected with the SNAP-25A-Myc-pEGFP.C1 plasmid where the cDNA encoding GFP was excised by digestion with *Nhe*I and *Sac*I. The plasmid was religated after refilling the 3' recessed ends with DNA Polymerase I (Klenow) fragment (Promega, Madison WI). Transfection of AtT-20 cells was done using LipofectAMINE and following the manufacturer's instructions. Stably transfected colonies were selected by growth in presence of neomycin (Calbiochem, San Diego, CA) used at a concentration of 0.75 mg/ml. Colonies were tested for expression of exogenous Myc-tagged SNAP-25 by Western blot with SNAP-25 antibody.

Secretion Assay

N2A cells grown in 60-mm dishes were transiently transfected with POMC-β-Gal and the indicated plasmids 72 h before the secretion experiments. For the experiment, cells were washed with secretion medium containing 20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.1 mg/ml bovine serum albumin, and incubated at 37°C for 1 h in 1 ml of the same medium with 5 mM glucose with or without 5 μM ionomycin. The medium was collected and centrifuged at 300 × g for 5 min to remove cell debris. The cell-free medium was incubated with 4 ml of acetone overnight at -20°C, centrifuged at 7000 × g for 15 min, loaded onto a SDS-PAGE gel, and analyzed by Western blot by using an antibody against β-Gal and Calnexin. At the end of the secretion experiment, the cells were scraped from plates in Kglu buffer, passed three times through a 27-gauge 1/2 syringe needle, and centrifuged at 100 × g for 5 min. The postnuclear supernatant was centrifuged at 7200 × g for 10 min to obtain P and SII fractions that were analyzed by Western blot with antibodies against β-Gal, Na⁺/K⁺ ATPase, SNAP-23, and Myc.

RESULTS

SNAP-23 Reconstitutes Ca²⁺-dependent Docking of Granules to Plasma Membranes Treated with BoNT/E

The cell-free docking assay was carried out using sealed inside-out plasma membrane ghosts isolated from either

AtT-20 or N2A (fraction P) together with endocrine granules (fraction SII) isolated from N2A cells transfected with a secretory chimera, POMC-β-Gal (Figure 1, A–C). POMC-β-Gal, synthesized as a 150-kDa protein, is processed in N2A granules to yield 137- and 120/124-kDa peptides (Koticha *et al.*, 2002). The extent of docking can therefore be revealed by the cosedimentation of the granules and plasma membrane with appearance in the blot of the processed 137- and 120/124-kDa-Gal immunoreactive bands.

Granule docking was abolished when AtT-20 plasma membranes were preincubated with botulinum toxin E (BoNT/E), which cleaves specifically SNAP-25. Thus, SNAP-25 is required for granule docking (Chieriegatti *et al.*, 2002; Washbourne *et al.*, 2002). When, in contrast, the docking reaction was carried out with plasma membranes from cells expressing exogenous SNAP-23, the process was unaffected by the toxin (Figure 1A). SNAP-23 is less sensitive than SNAP-25 to BoNT/E proteolysis (Washbourne *et al.*, 1997). In our experimental conditions (500 nM BoNT/E), the toxin did not cleave SNAP-23, whereas most of SNAP-25 was proteolyzed (Figure 1B). Some SNAP-25 (< 20%) may remain uncleaved because a fraction of AtT-20 plasma membranes does not expose the inner surface to the medium (Chieriegatti *et al.*, 2002). These data show that overexpressed SNAP-23 reconstitutes granule docking to AtT-20 plasma membranes in the presence of BoNT/E. The SNAP-23-dependent granule docking, like that supported by SNAP-25 (Chieriegatti *et al.*, 2002), is reversible because addition of 20 mM EGTA to chelate free Ca²⁺ induced release of the docked vesicles in the supernatant (Figure 1A).

Ca²⁺-dependent, Reversible, Docking of Granules Is Supported by Formation of a Syt1–SNAP-25 Complex without VAMP-2 and Syntaxin 1

Temperatures below 15°C inhibit fusion of biological membrane in cell-free assays because the process requires membrane fluidity (Latterich *et al.*, 1995; Brickner *et al.*, 2001). Because docking requires protein interactions rather than

Figure 2. The Ca^{2+} -dependent docking complex includes Syt1 and SNAP-25, but not syntaxin 1 and VAMP-2. (A) Fractions containing plasma membrane (P) and granule (SII) were derived from wild-type N2A cells (N2A). The plasma membrane-containing P fraction was incubated for 30 h at 30°C with or without 100 nM BoNT/C in a buffer containing 100 mM NaCl, 100 mM KCl, 10 mM HEPES, and 1 mM dithiothreitol. P membranes were centrifuged at $7200 \times g$ for 10 h, resuspended in Kglu buffer, mixed with granules in SII, and further incubated in the absence and in the presence of 10 μM free Ca^{2+} to induce docking. All samples were diluted in the IP buffer containing 50 μM free Ca^{2+} (see MATERIALS AND METHODS). Samples were immunoprecipitated with mouse monoclonal anti-Syt1 antibodies (p65) and analyzed by Western blot with antibodies against Syt1, SNAP-25, syntaxin, and VAMP2. Equal volumes of P and SII from cells were also analyzed (total). A representative experiment of a total of three independent ones is shown. (B) Plasma membranes in fraction P from N2A cells were incubated with or without BoNT/C as in A and mixed with granules derived from N2A transiently transfected with POMC- β -Gal. Samples were incubated at 30°C in the presence or absence of 10 μM free Ca^{2+} , and where indicated, with 1 mM ATP and 1 mM MgCl_2 (ATP). Samples were further incubated with or without 20 mM EGTA and analyzed as in Figure 1A. The entire pellet and one-half the supernatants were loaded into the SDS-PAGE acrylamide gel (C) Granules in fraction SII derived from N2A transiently transfected with POMC- β -Gal were incubated with and without 150 nM TeNT for 30 min at 30°C . The same fraction of each sample was analyzed by Western blot with antibodies against VAMP-2. The remaining TeNT-treated granules were mixed with the plasma membrane-containing fraction P from N2A cells and docking was induced and analyzed as in Figure 1C. Experiments in B and C were done twice, with similar results.

lipid mixing, it is expected that the process can also take place at low temperatures. In the presence of Ca^{2+} , granules were able to cofractionate with N2A plasma membranes (or AtT-20 plasma membranes; our unpublished data) at 4°C as well as at 30°C , indicating that docking, not fusion, has occurred (Figure 1C).

We have proposed that granule docking is supported by the interaction of Syt1 with SNAP-25 and without VAMP-2 (Chieriegatti *et al.*, 2002). To determine whether VAMP-2 and syntaxin 1 are excluded from the complex that supports docking, we did coimmunoprecipitation experiments with plasma membranes derived from N2A cells that express abundant VAMP-2 and syntaxin 1. In the presence of Ca^{2+} , SNAP-25 coimmunoprecipitated with Syt1, whereas syntaxin 1 and VAMP-2 did not (Figure 2A, lane 2). This indicates that syntaxin 1 and VAMP-2 do not bind to the Syt1-SNAP-25 complex in the docking condition.

We further explored a possible role of syntaxin 1 in docking by using BoNT/C. BoNT/C blocks neurotransmission by cleaving syntaxin 1 (Blasi *et al.*, 1993; Schiavo *et al.*, 1995). It has also been reported that BoNT/C cleaves SNAP-25, but only in intact cells and not in vitro (Foran *et al.*, 1996; Williamson *et al.*, 1996). In our assay, pretreatment of N2A

plasma membranes with BoNT/C lead to a reduced intensity of membrane-bound syntaxin 1 immunoreactive bands, whereas the SNAP-25 band was unchanged (Figure 2A, lanes 5 and 6). This indicates that most of syntaxin 1, but not SNAP-25 is cleaved in the assay. Syntaxin 1 proteolysis did not inhibit Ca^{2+} -dependent granule docking (Figure 2B, lane 3), indicating that the protein is not involved at this step. TeNT inhibits regulated exocytosis by cleaving VAMP-2 (Schiavo *et al.*, 1992). Granules treated with 150 nM TeNT (or with 450 nM TeNT; our unpublished data) had 70% less VAMP-2 than the untreated granules. The TeNT-treated granules docked efficiently to the plasma membranes (Figure 3C), indicating that VAMP-2 is not involved in docking. These experiments and the immunoprecipitation experiments described above indicate that Ca^{2+} -dependent granule docking is supported by formation of the SNAP-25-Syt1 complex without syntaxin1 and VAMP-2 (Figure 2).

The SNARE complex components SNAP-25, syntaxin 1, and VAMP-2 are required for regulated exocytosis. We reasoned that syntaxin 1 and VAMP-2 may associate to the Syt1-SNAP25 complex at stages that occur later than the reversible granule docking. When ATP was added to the docking assay in addition to Ca^{2+} , VAMP-2 and syntaxin 1

Figure 3. SNAP-23-dependent granule docking functions at lower $[Ca^{2+}]$ than that supported by SNAP-25. (A) Granule docking to plasma membranes from wild-type AtT-20 cells (SNAP-25-dependent docking) and to plasma membranes from AtT-20 cells expressing SNAP-23 treated with BoNT/E (SNAP-23-dependent docking) in the presence of the indicated free $[Ca^{2+}]$ was measured as in Figure 1A. (B) Three experiments, including that shown in A, are analyzed. Columns are mean OD values of the 120/124-kDa band of processed POMC- β -Gal $\pm$ SD. (C) Plasma membrane-containing fraction P from AtT-20 cells (AtT-20) and AtT-20 cells expressing SNAP-25A-Myc (AtT-20/SNAP-25, stable transfection) were analyzed by Western blot with antibodies against SNAP-25. The arrow indicates exogenous SNAP-25A-Myc protein. (D) Granule docking to plasma membranes from wild-type AtT-20 cells and to plasma membranes from AtT-20 cells expressing SNAP-25 in the presence of the indicated free $[Ca^{2+}]$ was measured as in A.

coimmunoprecipitated with Syt1-SNAP-25 (Figure 2A, lane 3). Proteolysis of syntaxin 1 by incubation with BoNT/C abolished coimmunoprecipitation of VAMP-2 with Syt1-SNAP-25 (Figure 2A, lane 4). These experiments indicate that both syntaxin 1 and VAMP-2 interact with the Syt1-SNAP-25 in the presence of Ca^{2+} and ATP. We have proposed that addition of ATP and Ca^{2+} (but not ATP by itself) to the assay induces irreversible association of granules with the plasma membrane (Chieriegatti *et al.*, 2002). In agreement with this conclusion, in the presence of Ca^{2+} and ATP, granules could not be released in the supernatant by subsequent addition of EGTA (Figure 2B, lane 6). However, when samples were pretreated with BoNT/C, granules failed to associate irreversibly with the plasma membranes (Figure 2B, lane 7). These experiments indicate that the Ca^{2+} and ATP-dependent association of granules with the plasma membrane, unlike granule docking, is supported by the formation of a complex that includes in addition to SNAP-25 and Syt1, also syntaxin 1 and VAMP-2.

SNAP-25 and SNAP-23-dependent Granule Docking Occurs at High and Low $[Ca^{2+}]$, Respectively

The data shown above indicate that SNAP-25-dependent, reversible docking of granules to the plasma membrane induced by Ca^{2+} is supported by the formation of the Syt1-SNAP-25 complex with the exclusion of other SNARE components. In terms of Ca^{2+} , docking to plasma membranes with exogenous SNAP-23 was already appreciable at 10^{-7} M $[Ca^{2+}]$ (Figure 3, A, B, and D). Differently, docking to plasma membranes with endogenous (Figure 3, A and B) or overexpressed SNAP-25 (20–50-fold higher protein level than

endogenous SNAP-25) (Figure 3, C and D) required $[Ca^{2+}]$ of 10^{-6} M or above. We conclude that SNAP-23 is able to replace SNAP-25 in granule docking, and it does so at Ca^{2+} concentrations 1 order of magnitude lower than SNAP-25-dependent docking.

The C2AB Domain of Syt1 Inhibits SNAP-25, but Not SNAP-23-dependent Docking

Synaptotagmins constitute a family of proteins with a single transmembrane domain, a linker, and a large cytoplasmic region composed of two domains that bind to phospholipids in a Ca^{2+} -dependent manner (C2A and C2B) (Sudhof, 2001; Chapman, 2002). Syt1 and the highly homologous Syt2 are thought to function as Ca^{2+} sensors in neurotransmission. The role of other synaptotagmins is less defined. Differently than in previous reports (Ullrich *et al.*, 1994; Li *et al.*, 1995), it has been recently proposed that synaptotagmins exhibit distinct Ca^{2+} affinities because Syt3, Syt7, and Syt5 were found to bind Ca^{2+} with ~ 10 -fold higher apparent affinity than Syt1 (Shin *et al.*, 2002; Sugita *et al.*, 2002).

On the basis of these data and of our observation that SNAP-25 and Syt1 function in Ca^{2+} -dependent granule docking, we reasoned that the different Ca^{2+} dependence of the SNAP-25- and SNAP-23-dependent docking processes could be due to their interaction with specific members of the synaptotagmin family. This possibility was first investigated by carrying out granule-plasmalemma docking assays in the presence of 5 μ M of the cytoplasmic domains (C2AB, with two Ca^{2+} -binding motifs) from the various members of the wide Syt family. For these experiments, granule docking was induced by 100 μ M free $[Ca^{2+}]$ instead of the 10 μ M

Figure 4. Syt1 C2AB inhibits specifically SNAP-25-dependent docking. (A and B) Plasma membranes from AtT-20 cells and from AtT-20 cells expressing SNAP-23 were pretreated with or without BoNT/E, mixed with granules, and incubated with 5 μ M GST-Syt1C2AB or GST-Syt1C2A (A) or with 5 μ M of the GST-C2AB domain of the indicated Synaptotagmins (B). Samples were further incubated in the absence or in the presence of 100 μ M Ca²⁺ to induce docking. Granules docked to the plasma membrane were analyzed by Western blot as in Figure 1A. (C) The table shows the effect of 5 μ M C2AB domains of the indicated Synaptotagmins and of 5 μ M Syt1C2A (C2A) on SNAP-23 and SNAP-25-dependent docking. Data were derived from experiments (n = 2 for each peptide) as in A and B.

[Ca²⁺] routinely used for the assay. This is because specific synaptotagmins that bind to several Ca²⁺ ions (five, for Syt1) may impair granule docking by lowering the [Ca²⁺] in the buffer.

By using this assay, it was found that the SNAP-25-dependent docking was completely inhibited by Syt1C2AB (Figure 4A). To test the specificity of the inhibition by Syt1C2AB, we used a shorter Syt1 protein that has only one Ca²⁺ binding motif (Syt1-C2A). Syt1C2A binds to phospholipids in Ca²⁺-dependent manner, but unlike Syt1C2AB binds weakly to SNAP-25 (Schiavo *et al.*, 1997; Gerona *et al.*, 2000; Earles *et al.*, 2001). Syt1C2A did not impair granule docking, indicating that Syt1C2AB acts specifically. Of the panel of C2AB fragments derived from the other Syt isoforms, two (Syt3 and Syt7) blocked SNAP-25-dependent docking to an extent similar to Syt1C2AB (Figure 4B); and two (Syt5 and Syt6; Figure 3C) were less effective (~40% inhibition). The other synaptotagmins (Syt4 and Syt11 [Figure 4B], and Syt8, Syt9, Syt10, and Syt12 [Figure 4C]) were inactive. Syt4 and Syt11 do not bind Ca²⁺ (von Poser *et al.*, 1997), and Syt8 and Syt12 lack the consensus Ca²⁺ binding site (Sudhof, 2001). The inability of these Syts to impair the SNAP-25-dependent granule docking is in agreement with the concept that this process is Ca²⁺ dependent. Similarly, SNAP-23-dependent docking was completely blocked by Syt3 and Syt7 and partially impaired by Syt5 and Syt6, whereas other synaptotagmins were inactive. Importantly, Syt1 failed to inhibit the SNAP-23-dependent granule docking (Figure 4A). These data indicate that SNAP-25 and SNAP-23 mediate docking by interacting with specific members of the synaptotagmin family. The data also support a model where SNAP-25 and SNAP-23 docking complexes

with specific synaptotagmins are triggered at different Ca²⁺ levels.

The Syt1-SNAP-25 Complex Forms in Trans between the Granule and Plasma Membrane

To study the role of Syt1 in granule docking, we first determined whether the Syt1-SNAP-25 complex forms in *trans* between the granule and plasma membrane. Docking experiments were carried out by mixing fractions from Syt1-YFP-transfected and nontransfected cells. If Syt1 on the granule establishes a complex with SNAP-25 at the plasma membrane, then the complex would form only when the fluorescent protein is delivered to the assay in the granule-containing fraction SII. Indeed, Syt1-YFP in the granule-containing fraction SII from transfected cells formed Ca²⁺-dependent complexes with SNAP-25 when incubated with the plasma membrane-containing fraction P from untransfected N2A cells (Figure 5A, lanes 5 and 6). Complexes did not form in the absence of added P membranes (Figure 5A, lanes 7 and 8), indicating that Syt1-YFP on the granule interacts only with SNAP-25 at the plasma membrane. Syt1-YFP in the plasma membrane-containing fraction P did not form complexes with SNAP-25 in the absence or in the presence of added granules (Figure 5A, lanes 1–4). These results indicate that the complex is indeed formed between the Syt1 on the granule and SNAP-25 at the plasma membranes.

Syt1 Forms Docking Complexes with Both SNAP-25 and Exogenous Syt7 (or Syt3) to Support Granule Docking to the Plasma Membrane

The structure of the SNAP-25-dependent complex could, however, be more complex than considered so far. In fact,

Figure 5. SNAP-25-dependent docking complex is formed in *trans* by SNAP-25 at the plasma membrane and Syt1 on the granule. (A) Fractions containing plasma membrane (P) and granule (SII) were derived either from wild-type N2A cells (N2A) or from N2A cells transiently transfected with Syt1-YFP, as indicated. P and SII were mixed and incubated in the absence and in the presence of 10 μ M free Ca^{2+} to induce docking. All samples were diluted in the IP buffer containing 50 μ M free Ca^{2+} (see MATERIALS AND METHODS). Samples were immunoprecipitated with mouse monoclonal anti-GFP antibodies and analyzed by Western blot with rabbit anti-GFP antibodies (to detect Syt1-YFP) and with antibodies against SNAP-25, as indicated. Equal volumes of P and SII from cells transfected with Syt1-YFP were analyzed (total, right). (B–D) Plasma membranes and granules from N2A cells (B) and N2A cells transiently transfected with Syt3-YFP (C) and Syt7-YFP (D) were mixed and preincubated with or without 5 μ M GST-Syt1C2AB, 5 μ M GST-Syt3C2AB, and 5 μ M GST-Syt7C2AB. After incubation in the absence or in the presence of 100 μ M free Ca^{2+} to induce docking, samples were diluted in the IP buffer (as in A) and immunoprecipitated with the indicated antibodies. The immunoprecipitated material was analyzed by Western blot with Syt1 antibodies (p65, to detect endogenous Syt1) and GFP antibodies (to detect Syt3-YFP and Syt7-YFP) and SNAP-25 antibodies. Arrowhead in B shows the immunoprecipitated Syt1. (E) Plasma membranes and granules from N2A cells or N2A cells transiently transfected with Syt7-YFP were used for the docking reaction. Samples were immunoprecipitated with Syt1 antibodies (p65) and analyzed by Western Blot with the indicated antibodies.

inhibition of granule docking to plasma membranes by the C2AB domains of Syt3 and 7 (Figure 4) suggested the possibility that not only Syt1, but also these other Syts interact with SNAP-25 in the docking step. In support of this possibility, the C2AB of Syt3 and Syt7, like that of Syt1, inhibited the formation of the Syt1–SNAP-25 complex in the docking assay (Figure 5B). It has been reported that Syt3 and Syt7 are expressed in peptide-hormone secreting cells and are involved in granule release (Brown *et al.*, 2000; Gao *et al.*, 2000; Fukuda *et al.*, 2002). Because Syt3 and Syt7 were equally potent inhibitors of granule docking, we focused on these isoforms to determine whether they were involved in the SNAP-25-dependent docking complex. To carry out these experiments, we overexpressed YFP-tagged Syt3 and Syt7 in N2A cells. We reasoned that this approach would determine whether these proteins could participate in the formation of the SNAP-25-dependent docking complex. The YFP-tag was used for these experiments because YFP-Syt1, like the endogenous Syt1, was already known to form docking complexes with SNAP-25 (Figure 5A). This observation indicated to us that the tag does not interfere with the docking process.

SNAP-25 coprecipitated with Syt3-YFP (or Syt7-YFP), showing that these proteins indeed interact with SNAP-25 in the docking condition (Figure 5, C and D). Moreover, the C2AB domain of Syt1, Syt3, and Syt7 inhibited formation of the Syt1–SNAP-25, Syt3–SNAP-25, or Syt7–SNAP-25 com-

plexes (Figure 5, B–D), thus establishing a strong correlation between formation of these complexes and the SNAP-25-dependent granule docking (Figure 4). Like the Syt1–SNAP-25 complex (Chieriegatti *et al.*, 2002), Syt3–SNAP-25 (our unpublished data) and Syt7–SNAP-25 complexes (Figure 5D) were dissociated when docked granules were released by lowering $[\text{Ca}^{2+}]$ in the assay. These data are consistent with the idea that exogenous Syt3 (or Syt7), like Syt1, interacts with SNAP-25 in a Ca^{2+} -dependent and reversible complex to support granule docking.

The data presented above also suggested that that exogenous Syt7 (or Syt3) might interact together with Syt1 and SNAP-25 in the same docking complex. When Syt1 was immunoprecipitated from samples incubated in the docking conditions, both SNAP-25 and Syt7-YFP were coimmunoprecipitated, indicating that these proteins participate in the same complex (Figure 5E).

It has been proposed that Syt7 and Syt3 are localized on granules (Brown *et al.*, 2000; Gao *et al.*, 2000; Fukuda *et al.*, 2002) and at the plasma membrane (Sugita *et al.*, 2001, 2002). Although Syt3-YFP (also Syt7-YFP) was expressed in the granule membrane fraction SII together with Syt1 (Figure 6, A and B, SII), an interaction between them could be established only in the presence of SNAP-25 in the plasma membrane fraction P (Figure 6, A and B, compare lanes 6 and 8). This experiment shows that Ca^{2+} induces the formation of a

Figure 6. Syt3 and Syt7 in the plasma membrane and granule fraction function in SNAP-25-dependent docking. (A and B) Fractions containing plasma membrane (P) and granule (SII) were derived from wild-type N2A cells (N2A) and cells transiently transfected with Syt3-YFP and with Syt7-YFP cDNA as indicated. P and SII were mixed and incubated in the absence and in the presence of 10 μ M free Ca²⁺ to induce docking. Samples were diluted in the IP buffer and immunoprecipitated as in Figure 5A. Samples were analyzed by Western blot with GFP antibodies (to detect Syt3-YFP and Syt7-YFP), with p65 antibodies (to detect endogenous Syt1), and with SNAP-25 antibodies, as indicated.

complex that includes Syt3 (or Syt7) and Syt1 on the granules and SNAP-25 at the plasma membrane.

Syt3-YFP (or Syt7-YFP) was able to interact with SNAP-25 also when located in the plasma membrane-containing fraction P, however, only in the presence of Syt1 in the granule membrane (Figure 6, A and B, compare lanes 4 and 2). We conclude that in the SNAP-25-dependent docking process the role of Syt3 and 7 is different from that of Syt1. In fact, it seems that the docking complex includes Syt1 on the vesicle, SNAP-25 at the plasma membrane and Syt3 or Syt7, no matter of their membrane localization.

SNAP-23 Forms Complexes with Exogenous Syt7 (or Syt3) but Not with Syt1, to Support Granule Docking to the Plasma Membrane

It remained to be established whether, and to what extent, the SNAP-23-dependent docking complex is structured as the SNAP-25-dependent one. This was hard to establish by using N2A cells because of their high endogenous expression of SNAP-25. We therefore used a N2A clone, G14, stably transfected with BoNT/E, in which most endogenous SNAP-25 is cleaved to inactive peptides (Koticha *et al.*, 2002). P and SII fractions isolated from G14 cells transfected with SNAP-23 together with Syt7, or Syt3 or Syt1, all coupled to YFP, were mixed in the docking assay, solubilized, and immunoprecipitated with antibodies against SNAP-23 (Figure 7A). Both Syt3 and Syt7, but not Syt1 (either the YFP-tagged exogenous or the endogenous protein), were found to coimmunoprecipitate with SNAP-23 in the docking condition. This experiment indicates that SNAP-23-dependent granule docking is supported by formation of a complex between SNAP-23 and Syt7 (or Syt3), with the exclusion of Syt1.

When added to the docking assay, the C2AB domain of Syt3 and Syt7 inhibited formation of the SNAP-23-Syt7 (or SNAP-23-Syt3) docking complexes (Figure 7, B and C). This observation is in agreement with the concept that the SNAP-23-Syt7 (or SNAP-23-Syt3) complex supports SNAP-23-dependent docking. Syt7C2AB and Syt3C2AB act specifically

because Syt1C2AB did not inhibit formation of the complexes. We conclude that the general structure of the SNAP-23-dependent complex may resemble that of its SNAP-25-dependent counterpart, with the important exclusion of Syt1.

Syt7 Is Expressed in AtT-20 and N2A Cells

To determine whether Syt3 and Syt7 are expressed in AtT-20 and N2A cells we used recently developed antibodies raised against the two proteins (Tucker *et al.*, 2003). We found that Syt7 is expressed both in AtT-20 and N2A cells, whereas Syt3 was not detectable in either of the two cell lines (Figure 8). We conclude that, in these cells, endogenous Syt7, rather than Syt3, is likely to participate in SNAP-25- and SNAP-23-dependent granule docking to the plasma membrane.

Expression of SNAP-23 in N2A Cells Leads to a 20-Fold Increase in Granule Exocytosis in the Basal Condition

The data shown in Figure 3 show that SNAP-25 and SNAP-23 function in granule docking at high and low Ca²⁺, respectively. We reasoned that if vesicle docking is the rate-limiting step of hormone release in the basal state, then overexpression of SNAP-23 could lead to enhanced granule release by resting cells. To investigate this possibility, intact N2A cells were transfected with the secretory chimera POMC- β -Gal and either control plasmid, SNAP-25, or SNAP-23. In cells expressing only endogenous or transfected SNAP-25, the release of the processed, granule-stored 120/124-kDa POMC- β -Gal was almost inappreciable at rest and increased moderately (fourfold) after administration of the Ca²⁺ ionophore ionomycin. In contrast, when cells were transfected with SNAP-23, release was considerable (20-fold higher) already at rest, and ionomycin induced no major increase (Figure 9, A and B). Consistently, the amount of 137- and 120/124-kDa peptides recovered in the granule-rich fraction SII was lower (21%) in SNAP-23-overexpressing than in mock-transfected cells, whereas the 150-kDa precursor, present in the endoplasmic reticulum (ER) and Golgi recovered in fraction P, was decreased to 71%. Other,

Figure 7. Interaction of SNAP-23 with Syt7 (or Syt3), but not with Syt1, supports SNAP-23-dependent granule docking. (A) Fractions containing plasma membrane (P) and granule (SII) were derived from G14 cells transiently cotransfected with SNAP-23 and Syt1-YFP or Syt3-YFP or Syt7-YFP and mixed in the docking assay. Incubations were carried out as in Figure 5B. Samples were immunoprecipitated with anti-SNAP-23 polyclonal antibody (Koticha *et al.*, 1999) and analyzed by Western blot with antibodies against GFP (to detect Syt1-YFP, Syt3-YFP, and Syt7-YFP) and p65 (to detect endogenous Syt1). (B and C) Plasma membranes (P) and granules (SII) from N2A cells transiently transfected with Syt7-YFP (B) and Syt3-YFP (C) were mixed and incubated with or without 5 μ M GST-Syt1C2AB, 5 μ M GST-Syt3C2AB, 5 μ M GST-Syt7C2AB, and 100 μ M Ca²⁺ as indicated. Samples were immunoprecipitated and analyzed by Western blot as in A.

nongranular markers (calnexin for the ER and Na⁺/K⁺ ATPase for the plasma membrane) were unchanged, showing that comparison had been carried out between similar numbers of cells. In conclusion, our data in intact cells demonstrate that SNAP-23 functions in granule release, but it does so at resting Ca²⁺.

It is possible that the biological effects induced by SNAP-23 expression on secretion are due to very high levels of the exogenous protein. Because parallel transfections with SNAP-25 had no effect on granule release, we determined the relative level of expression of exogenous SNAP-25 and SNAP-23 proteins in our assay by using GFP-tagged proteins as reference. Western blot analysis using anti-GFP antibodies shows that SNAP-25-GFP was approximately threefold more abundant than SNAP-23-GFP (Figure 9C). By using antibodies against SNAP-25, it seems that the SNAP-25-Myc protein without the GFP-tag (the same as used for the secretion studies in Figure 9A) was threefold more abundant than GFP-SNAP-25-Myc and 20- to 50-fold more abundant than endogenous SNAP-25 (Figure 9D). Differently, by using anti-SNAP-23 antibodies, it is found that SNAP-23 without the GFP tag was ~10-fold less abundant than GFP-SNAP-23 (Figure 9E). Thus, exogenous SNAP-23 in N2A cells is less abundant (<10-fold) than exogenous SNAP-25 and seems to be expressed at levels comparable with those of endogenous SNAP-25.

Expression of the C2AB Domain of Syt1 in N2A Cells Inhibits the SNAP-25-, but Not the SNAP-23-dependent Release of Granules

In N2A cells, SNAP-25 activity is necessary for regulated granule release (Koticha *et al.*, 2002). We have shown here that Syt1, the proposed Ca²⁺ sensor (Perin *et al.*, 1990; Brose *et al.*, 1992), is specifically required for SNAP-25-dependent docking of granules, a process that occurs at high [Ca²⁺]. On the basis of these considerations, we hypothesized that expression of the C2AB domain of Syt1 in N2A cells could lead to an inhibition of regulated hormone release by engaging SNAP-25 in an unproductive interaction. We also predicted

that Syt1C2AB would not impair the SNAP-23-dependent granule release in resting cells because SNAP-23-dependent docking does not require Syt1. Figure 10 shows that regulated release of granules was abolished by expression of Syt1C2AB. Differently, the SNAP-23-dependent release of the POMC- β -Gal peptides by resting cells was unchanged by expression of Syt1C2AB. Thus, Syt 1 is specifically involved in SNAP-25-dependent granule release. Expression of the C2AB domain of Syt7 in N2A cells inhibited both SNAP-25- and SNAP-23-dependent granule release (Figure 10). These data are in agreement with the observation that the C2AB domain of Syt7 inhibits both SNAP-25- and SNAP-23-dependent granule docking.

DISCUSSION

By using an *in vitro* assay, we have recently characterized docking of granules to the plasma membrane as a reversible step that is induced by Ca²⁺ and is supported by the formation of a complex that includes Syt1 on the vesicle and SNAP-25 at the plasma membrane (Chieriegatti *et al.*, 2002). Addition of ATP to the system changes the reversible interaction of the granules with the plasma membrane into an irreversible step that may correspond to later stages of docking or fusion. The irreversible step, but not reversible docking, requires the association of syntaxin 1 and VAMP-2 with the SNAP-25-Syt1 complex (Figure 2). Thus, by excluding ATP from the assay, it is possible to dissect a Ca²⁺-dependent docking step that is supported by the Syt1-SNAP-25 complex without participation of other SNARE components. It has been proposed that the C terminus of SNAP-25 is essential for Ca²⁺-dependent binding of Syt1 to SNAP-25 and for regulated exocytosis (Gerona *et al.*, 2000). The data presented here and in our earlier work (Chieriegatti *et al.*, 2002) indicate that this interaction is required for Ca²⁺-dependent docking of granules to the plasma membrane.

Here, we propose that SNAP-23 and SNAP-25 function in granule docking and release at low and elevated [Ca²⁺], respectively, by interacting with different members of the

Figure 8. Syt7 is expressed in AtT-20 and N2A cells. The standards (Std) correspond to 1.5 ng of a fragment of Syt 7 (residues 1–260) or 15 ng of a fragment of Syt 3 (residues 1–423) fused to GST, and immunoblot analysis was carried out using isoform specific antibodies as described in Tucker *et al.* (2003). The indicated amounts of postnuclear supernatants derived from AtT-20 cells, N2A cells, and brain were mixed with sample buffer and loaded onto the SDS gel.

Synaptotagmin family. These conclusions are based on several observations. First, by using the *in vitro* docking assay, it was possible to directly compare the Ca^{2+} sensitivity of SNAP-25- and SNAP-23-dependent docking of granules to plasma membranes. It was found that the SNAP-25-dependent process occurs at high $[\text{Ca}^{2+}]$ ($\geq 1 \mu\text{M}$), whereas SNAP-23-dependent docking occurs at 10-fold lower $[\text{Ca}^{2+}]$. Second, by using a panel of 11 different SytC2AB domains it is found that Syt1, Syt3, and Syt7 block specifically SNAP-25-dependent docking, whereas Syt3 and Syt7, but not Syt1, abolish the SNAP-23-dependent process. Third, in the docking conditions, SNAP-25 forms complexes with overexpressed Syt1 and Syt7 (or Syt3), whereas SNAP-23 forms complexes with Syt7 (or Syt3), but not with Syt1. Fourth, the C2AB domains of Syt1, Syt7, and Syt3 inhibit both SNAP-25-dependent granule docking, formation of the complexes, and granule release. Importantly, the C2AB domain of Syt7 and Syt3, but not of Syt1, inhibits the corresponding SNAP-23-dependent processes.

The experiments presented in this study show that overexpressed Syt7 and Syt3 have largely overlapping function and participate both in SNAP-25- and SNAP-23-dependent granule docking. Endogenous Syt7 is expressed in N2A and AtT-20 cells, whereas Syt3 was not detected under our blotting conditions. Thus, in these cells, Syt7, rather than Syt3, is likely to participate in SNAP-25- and SNAP-23-dependent docking complexes. It has been reported that this protein is expressed in the β -cells of the pancreas and regulates insulin

secretion (Brown *et al.*, 2000; Gao *et al.*, 2000). In agreement with these reports, the data presented here suggest that Syt3 may interact with SNAP-25 and Syt1 to support docking and fusion of insulin-containing granules.

On the basis of the results presented here and by other investigators, a novel model is proposed to explain docking/fusion at low and high Ca^{2+} in endocrine cells (Figure 11). In resting cells, granules are positioned near the cell membrane (morphological docking) by Rab3- and Munc-18-dependent mechanisms (Baldini *et al.*, 1998; Martelli *et al.*, 2000; Voets *et al.*, 2001). In response to an increase in intracellular $[\text{Ca}^{2+}]$, granules interact reversibly with the plasma membrane (functional docking) by forming a complex that includes Syt1 on the granule, SNAP-25 at the plasma membrane and Syt7 (or Syt3) either on the granules or at the plasma membrane. This conclusion is based on our *in vitro* findings that 1) Syt1, Syt7, and SNAP-25 form complexes that support granule docking at $1 \mu\text{M}$ Ca^{2+} ; 2) both granule and plasma membrane-associated Syt7 (or Syt3) interact with granule-bound Syt1 and plasma membrane-bound SNAP-25 to form the docking complex (Figures 5 and 6).

The model predicts that defects in Syt1, Syt7 and SNAP-25 proteins would affect functional granule docking induced by elevated $[\text{Ca}^{2+}]$ and not granule positioning near the plasma membrane (also referred to as morphological docking) at resting $[\text{Ca}^{2+}]$ levels. Thus, the model is consistent with the finding that in the SNAP-25 mutant mouse, there are no defects in accumulation of synaptic vesicles at the synapse or in the positioning of granules near the plasma membrane (Washbourne *et al.*, 2002; Sorensen *et al.*, 2003). However, it is possible that Syt1 is involved in vesicle docking to the plasma membrane at resting Ca^{2+} levels, perhaps by interacting with other plasma membrane components. This is suggested by the finding that the pool of docked synaptic vesicles is severely reduced in *Synaptotagmin* mutants of *Drosophila* (Reist *et al.*, 1998).

In the model, Syt1 acts as a low-affinity Ca^{2+} sensor that regulates SNAP-25-dependent granule docking. This model is based on the observation that Syt1 binds to Ca^{2+} with a lower apparent affinity than other synaptotagmins (Shin *et al.*, 2002; Sugita *et al.*, 2002), that SNAP-25-dependent granule docking is triggered by elevated $[\text{Ca}^{2+}]$ ($\geq 1 \mu\text{M}$), and that Syt1 is specifically required for the formation of the SNAP-25-dependent docking complex (Chiergatti *et al.*, 2002; this article). In the model, it is predicted that, after docking, additional ATP- and Ca^{2+} -dependent steps lead to the formation of the SNARE complex and fusion (Klenchin and Martin, 2000).

In agreement with the *in vitro* data showing that SNAP-23 supports granule docking at low Ca^{2+} , it is found that overexpressed SNAP-23 leads to an increase of granule exocytosis in the absence of elevated intracellular Ca^{2+} . This observation indicates that the ability of endocrine cells to store granules at resting $[\text{Ca}^{2+}]$ and, therefore, to release them in response to elevated Ca^{2+} , is controlled by SNAP-23 expression level. The high apparent Ca^{2+} affinity of Syt7 and Syt3 and their ability to function in the SNAP-23-dependent docking in the absence of Syt1 can explain for the first time how both the docking and the fusion steps of regulated exocytosis, a process classically known as Ca^{2+} -triggered, can take place at resting $[\text{Ca}^{2+}]$.

Studies in permeabilized insulinoma cells suggested that SNAP-23 replaces, albeit less efficiently, SNAP-25 activity in hormone secretion (Sadoul *et al.*, 1995). More recently, it has been reported that in chromaffin cells of SNAP-25 null mice, exogenous SNAP-23 competes with SNAP-25 for participa-

Figure 9. Expression of SNAP-23 in N2A cells leads to high levels of hormone release in the basal state. (A) N2A cells were transiently cotransfected with *POMC-β-Gal-pcDNA3* and control *pcB7* vector (mock), SNAP-23-*pcB7* (SNAP-23), or SNAP-25A-*Myc-pcB7* (SNAP-25). Cells were kept in basal and stimulated (+ 5 μM Ionomycin) conditions as described in Secretion Assay in MATERIALS AND METHODS. The medium and the P and SII fractions derived from the cells were analyzed by Western blot with the indicated antibodies. (B) Three independent experiments including that shown in B were analyzed. Columns are mean OD values of the 120/124-kDa band of processed POMC-β-Gal in the medium ± SD. (C–E) In parallel experiments N2A cells were transfected with SNAP-25A-*Myc-pEGFP.C1* (GFP-SNAP-25) (C and D), SNAP-23-*pEGFP.C1* (GFP-SNAP-23) (C and E), SNAP-25A-*Myc-pcB7* (SNAP-25) (D), and SNAP-23-*pcB7* (SNAP-23) (E). Equal amounts (10 μg) of P fractions derived from these cells were analyzed with antibodies against GFP, SNAP-23, and SNAP-25, as indicated. (D and E) Same blot exposed for 20 s (left) and 4 min (right).

tion in granule secretion, but does not support an initial exocytotic burst (Sorensen *et al.*, 2003). Strikingly, the expression of SNAP-23 in control cells induced a decrease in the burst and overall secretion, indicating that a population of primed vesicles is depleted by expression of SNAP-23. However, in this study, the effect of SNAP-23 expression on granule release at low levels of Ca^{2+} was not investigated, so the possibility exists that secretion is inhibited by depletion of exocytotic vesicles that are efficiently released at resting Ca^{2+} levels. This possibility is indeed supported by our observation that the general pool of granules is severely depleted in N2A cells that overexpress SNAP-23, as expected by their enhanced ability to secrete these vesicles in resting conditions.

Recruitment of new vesicles able to fuse with the plasma membrane is a process that occurs both constitutively and in a regulated manner. Regulated exocytosis, a function classically attributed to neurons, endocrine and exocrine cells, is now recognized as a widespread and possibly ubiquitous cellular property (Dan and Poo, 1992; Chavez *et al.*, 1996; Borgonovo *et al.*, 2002). The processes taking place in cells of different type are, however, profoundly different. The extensive work carried out on synaptic vesicle fusion has revealed fundamental aspects that probably have a general importance, adapted, however, to the diverse needs of the other cells. The nature and extent of these adaptations are not well understood. SNAP-23 had already been shown to be involved in the translocation of Glut4 vesicles (Rea *et al.*, 1998; Foster *et al.*, 1999; Kawanishi *et al.*, 2000), an insulin-induced

Figure 10. C2AB domain of Syt1 inhibits regulated exocytosis but does not impair SNAP-23-dependent granule release in the basal state. (A) N2A cells were transiently cotransfected with *POMC-β-Gal-pcDNA3*, SNAP-25A-*Myc-pcB7* (SNAP-25), and *pcDNA/GW/b-TOPO-Syt1C2AB-EYFP* (Syt1C2AB) or *pcDNA/GW/b-TOPO-Syt7C2AB-EYFP* (Syt7C2AB). Cells were kept in basal and stimulated (+ 5 μM Ionomycin) conditions and secretion was measured as in Figure 8. (B) N2A cells were transiently cotransfected with *POMC-β-Gal-pcDNA3*, SNAP-23-*pcB7* (SNAP-23), and *pcDNA/GW/b-TOPO-Syt1C2AB-EYFP* (Syt1C2AB) or *pcDNA/GW/b-TOPO-Syt7C2AB-EYFP* (Syt7C2AB), and secretion was analyzed as in A. Columns are mean OD values of the 120/124-kDa POMC-β-Gal band in the medium (derived from three independent experiments) shown as percentage of the ionomycin-treated sample ± SD.

Figure 11. SNAP-25- and SNAP-23-dependent docking/fusion of granules. In endocrine cells with endogenous levels of SNAP-25 and SNAP-23 granules positioned at the cell cortex dock to the plasma membrane in response to an increase in intracellular $[Ca^{2+}]_i$ by forming a complex with SNAP-25, Syt1, and Syt3 (or Syt7). This step is followed by other ATP- and Ca^{2+} -dependent steps that lead to SNARE complex formation and fusion. Differently, in endocrine cells overexpressing SNAP-23, granules dock and fuse at resting Ca^{2+} levels by forming a complex with SNAP-23 and Syt7.

form of vesicle exocytosis that takes place in adipocytes and does not need $[Ca^{2+}]_i$ changes. Other forms of regulated exocytosis are known to be independent of increases in $[Ca^{2+}]_i$ (Lorenz *et al.*, 2003). Based on our data, the hypothesis can be put forth that they function via the SNAP-23–Syt3 (or Syt7 or Syt5 or Syt6) interaction. In mast cell an increase in $[Ca^{2+}]_i$ supports compound exocytosis by relocating SNAP-23 from plasma membrane lamellipodia-like projections to granule membranes (Guo *et al.*, 1998). In other cells that express SNAP-23, but not SNAP-25, similar $[Ca^{2+}]_i$ -dependent relocation mechanisms may function to support regulated exocytosis. For example in fibroblasts, Syt7 has been implicated in Ca^{2+} -dependent exocytosis of lysosomes to repair plasma membrane wounds (Reddy *et al.*, 2001). SNAP-23 is expressed in fibroblasts and has been proposed to be associated with vimentin filaments that would act as a reservoir to supply the protein to the plasma membrane (Faigle *et al.*, 2000). More work is still needed to understand how the final steps of constitutive and regulated exocytosis work and how they are related. What is already clear, however, is that one, and possibly the most important mechanism by which the cell governs its regulated exocytotic process is its expression of SNAP-25, SNAP-23, and Syts.

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