Plasma membrane targeting of SNAP-25 increases its local concentration and is necessary for SNARE complex formation and regulated exocytosis

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Summary

SNAP-25 is an integral protein of the plasma membrane involved in neurotransmission and hormone secretion. The cysteine-rich domain of SNAP-25 is essential for membrane binding and plasma-membrane targeting. However, this domain is not required for SNARE complex formation and fusion of membranes in vitro. In this paper, we describe an 'intact-cell'-based system designed to compare the effect of similar amounts of membrane-bound and soluble SNAP-25 proteins on regulated exocytosis. In transfected neuroblastoma cells, Botulinum neurotoxin E (BoNT/E), a protease that cleaves SNAP-25, blocks regulated release of hormone. However, hormone release is rescued by expressing a wild-type SNAP-25 protein resistant to the toxin. BoNT/E-resistant SNAP-25 proteins lacking the cysteine-rich domain or with all the cysteines substituted by alanines do not form SNARE complexes or rescue regulated exocytosis when expressed at the same level as membrane-bound SNAP-25, which is approximately four-fold higher than the endogenous protein. We conclude that the cysteine-rich domain of SNAP-25 is essential for Ca^{2+}-dependent hormone release because, by targeting SNAP-25 to the plasma membrane, it increases its local concentration, leading to the formation of enough SNARE complexes to support exocytosis.

Key words: SNAP-25, Regulated exocytosis, Cysteine-rich domain

Introduction

Regulated exocytosis of neurotransmitters and hormones, as well as intracellular traffic, requires fusion of two lipid bilayers (Jahn and Sudhof, 1999). Biochemical and genetic evidence imply that SNARE proteins play a fundamental role in membrane fusion. SNARE proteins are thought to form a protein bridge, the SNARE complex, between an incoming vesicle and the acceptor compartment (Hanson et al., 1997; Sollner et al., 1993; Sutton et al., 1998). It has been proposed that formation of the SNARE complex promotes membrane fusion by bringing two separate lipid bilayers into close proximity. The SNARE components involved in neurotransmission are two integral membrane proteins of the plasma membrane, the t-SNAREs, Syntaxin-1 and SNAP-25 (Oyler et al., 1989), and a synaptic vesicle protein, the v-SNARE VAMP-2 (Sollner et al., 1993). Recent findings show that SNARE proteins contribute to the specificity of membrane fusion (McNew et al., 2000; Scales et al., 2000), implying that the mechanisms by which SNAREs are targeted to subcellular compartments are important for specific docking and fusion of vesicles.

SNAP-25 and its non-neuronal homologue Syndet/SNAP-23 (Wang et al., 1997; Ravichandran et al., 1996) are synthesized as soluble proteins in the cytosol. Others and ourselves have shown that both SNAP-25 and Syndet/SNAP-23 are palmitoylated at cysteine residues clustered in a loop between two N- and C-terminal coils and that palmitoylation is essential for membrane binding and plasma membrane targeting (Gonzalo et al., 1999; Gonzalo and Linder, 1998b; Koticha et al., 1999; Veit et al., 1996; Vogel et al., 2000). It is possible that palmitoylation at the cysteine-rich domain of SNAP-25 modulates its biological activity. However, it is not clear whether the cysteine-rich domain is necessary for function. The loop with the cysteine-rich motif is not required for complex formation in vitro (Fasshauer et al., 1998; Poirier et al., 1998; Vogel et al., 2000). Reconstitution experiments using liposomes show that the cysteine-rich domain is not necessary for vesicle fusion in vitro (Parlati et al., 1999). In cracked PC12 cells treated with BoNT/E toxin, bacterially expressed, non-palmitoylated, wild-type SNAP-25 and a full-length SNAP-25 mutant with its cysteines mutated into alanine residues are able to reconstitute Ca^{2+}-dependent exocytosis (Scales et al., 2000). In agreement with these findings, it has been suggested that soluble SNAP-25 mutants have biological activity and support secretion in Streptolysin-O-permeabilized HIT cells (Gonelle-Gispert et al., 2000). However, it has also been proposed that the cysteine residues of SNAP-25 are required for exocytosis in Streptolysin-O-permeabilized PC12 cells (Washbourne et al., 2001). The discrepancy between data using cracked or permeabilized cells may be caused by different levels of exogenous SNAP-25 in the transfected cell. Moreover, by using Streptolysin-O-permeabilized cells, it is difficult to establish whether soluble SNAP-25 proteins are indeed functional. This is because soluble SNAP-25, unlike the membrane-bound protein, can diffuse out of the pores created by toxin. In conclusion, although it is established that palmitoylation of SNAP-25 plays an important role in targeting the protein to the plasma membrane, the question of whether

this process is required for SNAP-25 function in exocytosis remains under discussion. To test whether membrane association and plasma-membrane targeting is required for function, we have used an 'intact-cell'-based system. In this system, the level of expression of exogenous SNAP-25 is similar to that of the endogenous protein, and the activity of the same amount of soluble and membrane-bound SNAP-25 can be directly compared. By using this controlled experimental system, we find that soluble SNAP-25 proteins do not form SNARE complexes and are unable to reconstitute regulated secretion.

Materials and Methods

Materials

Enzymes for DNA modification and restriction enzymes were purchased from Promega (Madison, WI). The pEGFP-C1 vector was purchased from Clontech (Palo Alto, CA). pcDNA3.1 (+), pIND, pIND expressing β-Gal (pIND/lacZ) and pVGRXR vectors along with Ponasterone A were purchased from Invitrogen (Carlsbad, CA). Lipofectamine was purchased from GIBCO BRL (Grand Island, NY). Mouse monoclonal anti-ACTH antibody was purchased from Cymbus Biotech (Hampshire, UK), and anti-Synaptobrevin 2 (VAMP-2) monoclonal antibody Cl 69.1 was a kind gift from Reinhard Jahn, Yale University (New Haven, CT) (Walch-Solimena et al., 1995). Anti-β-Gal and secondary peroxidase-conjugated anti-mouse and anti-rabbit IgG were purchased from Roche (Indianapolis, IN). Anti-myc 9E10 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Syntaxin-1 antibody, Ionomycin and O-Nitrophenyl-β-D-Galacto-pyranoside (ONPG) were purchased from Sigma (St. Louis, MO). Rabbit anti-Syndet/SNAP-23 antibody was raised as described previously (Wang et al., 1997). The mouse monoclonal anti-α1 subunit of Na^+/K^+ ATPase was from Upstate Biotechnology Inc (Lake Placid NY). Mouse monoclonal antibody 42.1 against Rab3 was provided by R. Jahn (Max-Planck-Institute for Biophysical Chemistry, Gottingen, Germany) (Matteoli et al., 1991). [Rabbit antibody against insulin-like growth factor II (IGF-II)/mannose 6-phosphate receptor Tanner (Tanner and Lienhard, 1989) was provided by G. E. Lienhard (Dartmouth Medical School, Hanover, NH)]. Enhanced chemiluminescence detection kits were purchased from NEN Life Science Products (Boston, MA).

Constructs

Murine POMC-pSP65 plasmid was a kind gift from Gary Thomas (Oregon Health Sciences University, Portland, Oregon). POMC-β-Gal was constructed by performing a PCR on POMC-pSP65 using the following primers: 5′ GGTGCTAGCCGCCTTTCCGCGACAGAG and 5′ TAAAAGCTTCTGGCCCTTCTTCTGCGC. The PCR product was cut with *Nhe*I and *Hin*dIII and inserted into the *Nhe*I- and *Hin*dIII-digested pcDNA3.1 vector to obtain POMC-pcDNA3.1. β-Gal cDNA was excised from *pIND/LacZ* using *Hin*dIII and *Xba*I, and this fragment was inserted into *Hin*dIII and *Xba*I-digested POMC-pcDNA3.1 to obtain POMC-β-Gal-pcDNA3.1.

SNAP-25A-pcB7 containing a myc-tag at the C-terminus was excised with *Kpn*I and *Bam*HI from SNAP25-Amyc-pCMX vector (Bark et al., 1995) and subcloned into the pcB7 vector (Koticha et al., 1999). Delta-SNAP25-A-myc-pcB7 was generated using the Long-Distance Inverse PCR method as described by Koticha et al. (Koticha et al., 1999) using SNAP-25A-pcB7 as a template and the following primers: 5′ AAACTTAAATCCAGTGATGCTTACAAAA and 5′ GCCTAAATCTTTTAAATTTTTCTCG. SNAP-25 protein encoded by SNAP-25A-pcB7 or Delta-SNAP-25A-pcB7 was made resistant to Botulinum Neurotoxin E (BoNT/E) by changing ^{179}Asp into a Lys residue and ^{182}Met into Thr residue using the following primers: 5′ GCATCACAGAGAAGGCTGACTCCAACAAAACCAG and 5′ GCTTAATCTGGCGATTCTGGGTGTCAATCTC and performing PCR as described above. SNAP-25A with its cysteines changed to alanines was generated by performing PCR on SNAP-25A/ER-pcB7 using the following primers: 5′ CGGCTAATAAACTTAAATCCAGTGATGCTTACAA and 5′ GCGCTATAAATAATCCTGCTGCTTTGCCTAAATCTTTTAAATTTTTCTC giving CA-SNAP-25A/ER-pcB7. SNAP-25A lacking the N-terminal helix and containing amino acids 82-206 was generated by performing PCR on SNAP-25A/ER-pcB7 using the following primers; 5′ TGGTAGTGGTGGGGGGTTG and 5′ TGGATTTAGGCAAATGCTGTGGC to give SNAP-25A/ER-82-206-pcB7. SNAP-25A lacking the N-terminal helix and the loop between the helices (amino acids 140-206) was generated by performing PCR on SNAP-25A/ER-pcB7 using the following primers; 5′ TGGTAGTGGTGGGGGGTTG and 5′ TGGATGCCCGGGAAAATGAAA to give SNAP-25A/ER-140-206-pcB7.

BoNT/E light chain was amplified from BoNT/E-pCMV plasmid (a kind gift from T. Binz, Hanover, Germany) using primers: 5′ CCTCCTGCGCTCGAGTCTAGATTACCTTATGCCTTTTACAGAA and 5′ TAATTAACCTAAGCTTGCCACCATGGGAATGCCAAAAATTAATAGTTTTAAT, digested with *Hin*dIII and *Xba*I and subcloned into pCDNA3.1 and pIND vectors to give BoNT/E-pCDNA3.1 and BoNT/E-pIND. Syndet/SNAP-23-Delta-BoNT/E-pcB7 has been described elsewhere (Koticha et al., 1999).

Cell culture and transfections

Neuro2A cells (a kind gift from Peter Cserjesi, Columbia University, NY) were cultured in DMEM with 8% FBS. Cells were transfected with Lipofectamine, according to the manufacturer's instructions. The efficiency of this method was determined by co-transfecting the pEGFP.C1 construct together with POMC-β-Gal and counting the percentage of cells expressing green fluorescent protein (GFP). Approximately 15% of Neuro2A cells expressed GFP when transfected with plasmids derived from DH5α bacterial cells. Neuro2A cell lines stably expressing BoNT/E, in the pIND vector, (G14 cells) were prepared according to the manufacturer's instructions. The G14 cells were routinely transfected with plasmids derived from JM109 bacteria to increase to 25% their tranfection rate. Cells were induced with 5 μM Ponasterone A for 20 hours before experiments.

Estimation of the amount exogenous SNAP-25 expressed in Neuro2A cells

To estimate the amount of Myc-tagged CA-SNAP-25A/ER expressed in Neuro2A cells, an SDS-PAGE gel was loaded with post-nuclear supernatants derived from 2 and 4×10^5 cells. The intensity of the protein band obtained by western blot analysis with anti Myc-antibodies was compared with that of known amounts (2.8, 5.6, 11.2, 22.4 and 44.8 ng) of Myc-tagged GFP-ensconsin (a kind gift from J. Chloe Bulinski, Columbia University, NY) (Faire et al., 1999). We estimated that approximately 0.0225 pg of CA-SNAP-25A/ER are expressed per transfected cell. Neuro2A cell lines stably expressing BoNT/E, in the pIND vector were prepared according to the manufacturer's instructions. Cells were induced with 5 μM Ponasterone A for 20 hours before experiments. The volume of Neuro2A cells was estimated by measuring confocal images of 22 cells using the 'LSM 510 Image Examiner' software (Zeiss, Thornwood, NY). The shape of these cells was approximated to that of an ellipsoid with diameters (μm) of: d_1=27.4±12, d_2=17.3±5.6 and d_3=6.15±1 and calculated to have a volume of approximately 1.5×10^{-6} μl per cell. Assuming that 50% of the cell volume is occupied by organelles and that the molecular weight of the Myc-tagged CA-SNAP-25A/ER is approximately 25 kDa, we deduced that the concentration of the exogenous, soluble SNAP-25 in the cytosol of the transfected cell corresponds to 1 μM.

Secretion assay

Neuro2A cells grown in 35 mm wells were transiently transfected with POMC-β-Gal and the indicated constructs 48 hours before the secretion experiments. For the experiment, cells were washed twice with M2 medium (145 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 0.7 mM $CaCl_2$ and 1 mg/ml BSA) and incubated at 37°C for 30 minutes in 1.0 ml M2. The medium was replaced with 1.0 ml M2 buffer with or without 1 μM Ionomycin for another 60 minutes. The medium was collected and centrifuged at 300 ***g*** for 5 minutes at room temperature to remove cell debris. Of this cell-free medium, 0.75 ml was added to 0.22 ml M2 containing 4 mg/ml ONPG. This reaction was incubated at 37°C for 60 minutes to measure β-Gal enzymatic activity. This incubation period was optimized so that the absorbance of the reactions was measured within the linear range. The reaction was stopped by the addition of 0.02 ml of 200 mM EGTA and 0.5 ml of 1 M Na_2CO_3. The absorbance was read at 420 nm. To measure total β-Gal activity in homogenates from transfected Neuro2A cell and G14 cell, cells were scraped in 0.250 ml M2 buffer and mixed with an equal volume of M2 buffer containing 2% Triton. After incubation on ice for 30 minutes, samples were centrifuged at 5000 ***g*** for 5 minutes. The β-Gal activity of 0.05 ml of the supernatant was measured as described above. Average cell β-Gal activities were obtained from triplicate samples per experiment. We estimated the fraction of damaged cells by measuring the amount of β-Gal activity released by Neuro2A cells expressing wild-type β-Gal after 1 hour of incubation in basal conditions. The β-Gal activity released was calculated as a percentage of the total β-Gal activity in the cell. This experiment was done twice, with triplicate points. For some experiments, cells were depolarized with 55 mM KCl in the presence of calcium. The NaCl concentration of the M2 medium was reduced to 85 mM, and KCl was increased to 55 mM. The cells were kept in this medium for 120 minutes. The medium was collected and processed as described above.

SNARE complex analysis

Neuro2A cells grown in a 35 mm plate were scraped in 0.5 ml of buffer containing 10 mM HEPES, 200 mM sucrose 10 mM EDTA and 2 mM EGTA, pH 7.4 and homogenized by six passages through an insulin needle as described previously (Koticha et al., 1999). The homogenates were centrifuged at 600 ***g*** for 5 minutes. The post-nuclear supernatant was mixed with an equal volume of sample buffer containing 4% SDS, sonicated for 2 seconds to disperse the pellet and electrophoresed on a 9% SDS-PAGE gel. A slice of the SDS-PAGE gel corresponding to the 99 kDa region was excised. The gel slice was heated at 100°C, re-electrophoresed on a 13% gel and analyzed by western blot with anti-SNAP-25, anti-VAMP-2 and anti-Syntaxin-1 antibodies as described (Otto et al., 1997).

Cell fractionation, electrophoresis procedures and confocal micrography

These procedures were carried out as described previously (Koticha et al., 1999). Post-nuclear supernatants were derived from N2A cell homogenates centrifuged at 600 ***g*** for 5 minutes. The protease inhibitor cocktail Complete Mini (Roche Diagnostics, Mannheim, Germany) was mixed with the homogenization buffer (20 mM Hepes pH 7.4, 120 mM potassium glutamate, 20 mM potassium acetate, 1 mM EGTA, 1 mg/ml BSA) before the experiment. For immunofluorescence, antibodies against SNAP-25 were used at a dilution of 1:2000 and antibodies against myc were used at a dilution of 1:400.

Results

POMC fused to a reporter protein is processed and targeted to the regulated secretory pathway in Neuro2A cells

GFP is targeted to secretory granules when fused to either human chromogranin B or insulin (Kaether et al., 1997; Lang et al., 1997; Pouli et al., 1998). This indicates that a hormone precursor can target a protein tag to the regulated secretory pathway. Neuro2A cells, a murine neuroblastoma cell line, which expresses exogenous POMC, cleaves POMC to yield β-lipoprotein and β-endorphin, targets these peptides to secretory granules and releases them in a Ca^{2+}-dependent manner (Chevrier et al., 1991; Noel et al., 1989). We prepared a cDNA encoding the chimera POMC-β-Gal (Fig. 1A). We reasoned that, by using this construct, processed POMC would be targeted to dense core granules, and hormone release in the medium could be detected by measuring β-Gal enzymatic activity with a spectrophotometric assay. To determine whether POMC can target a protein fused to its C-terminus to the regulated secretory pathway, we transiently expressed a POMC-β-Gal chimera in Neuro2A cells. Cells transfected with POMC-β-Gal plasmid express a protein of approximately150 kDa that is detected with both anti-ACTH and anti-β-Gal antibodies (Fig. 1A). This band corresponds to a fusion protein containing POMC (31 kDa) and β-Gal (120 kDa). The 150 kDa POMC-β-Gal fusion protein is processed into a 137 kDa band that is also detected with both anti-ACTH and anti-β-Gal antibodies and thus contains ACTH, β-lipotropin and β-Gal and lacks the N-terminal peptide of POMC. POMC-β-Gal is processed into two smaller peptides of approximately 124 kDa and 120 kDa (the two proteins are indicated by double arrows). Both of these peptides are not detectable with the anti-ACTH antibody. The 124 kDa peptide may contain either β-lipoprotein or β-endorphin fused to β-Gal. The 120 kDa peptide corresponds to β-Gal. POMC-β-Gal and its processed forms were all found in the membrane fraction, whereas 50% of wild-type β-Gal was in the cytosol (Fig. 1A). The β-Gal tag of POMC-β-Gal was not proteolyzed, indicating that peptide cleavage occurs specifically at the hormone precursor. Processing of POMC occurs when the precursor is packaged into secretory granules (Orci et al., 1987; Tooze et al., 1987). Thus, processing of POMC-β-Gal indicates targeting to a specialized compartment. To determine the relative distribution of the precursor and processed forms of POMC-β-Gal, the post-nuclear supernatant of the transfected N2A cells was centrifuged at 7,200 ***g*** for 10 minutes. The 150 kDa POMC-β-Gal precursor fractioned into the pellet P1, whereas the processed POMC-β-Gal bands at 137 kDa and 124/120 kDa were almost entirely collected in the supernatant S1 (Fig. 1B). This observation indicates that the precursor and processed forms of POMC-β-Gal are sorted into different compartments. The Rab3 immunoreactivity was recovered in S1 together with processed POMC-β-Gal. Others as wells as ourselves have shown by using cell fractionation and immunoelectron microscopy that Rab3 proteins localize to dense core granules (Baldini et al., 1998; Iezzi et al., 1999; Martelli et al., 2000). As granules are stored at the tips of Neuro2A processes (Chevrier et al., 1991), we asked whether Rab3 immunoreactivity accumulated in this region. In our culture conditions, approximately 30% of the Neuro2A cell population developed processes (Fig. 1C, arrows), whereas most of the cells were round (Fig. 2B,C). Rab3A immunoreactivity concentrated at the tip of the processes, indicating Rab3 protein localization to the granule (Fig. 1C). Unlike Rab3, most of the POMC-β-Gal immunoreactivity was accumulated at the cell body (data not shown). This observation indicates that a large

fraction of POMC-β-Gal immunoreactivity, perhaps the 150 kDa precursor, is not localized into the granule. To determine whether the processed forms of β-Gal and Rab3 co-fractionate, a 70,000 ***g*** pellet derived from fraction S was loaded onto a 20%-60% (w/v) sucrose density gradient (Fig. 1D). Rab3A and processed POMC-β-Gal co-migrated in the gradient, whereas the Mannose-6-Phosphate receptor, a protein localized in the trans-Golgi and late endosomes (Lombardi et al., 1993), concentrated in a lighter region. In conclusion, these experiments indicate that the processed forms of POMC-β-Gal, but not the 150 kDa precursor, are targeted to dense core granules.

To determine whether processed POMC-β-Gal is secreted in a regulated manner, cells were incubated for 1 hour in basal conditions and with Ionomycin in the presence of Ca^{2+}. The medium was analyzed by western blot (Fig. 1E). Results from three independent experiments indicated that the average ratio of the 150 kDa POMC-β-Gal precursor found in the medium

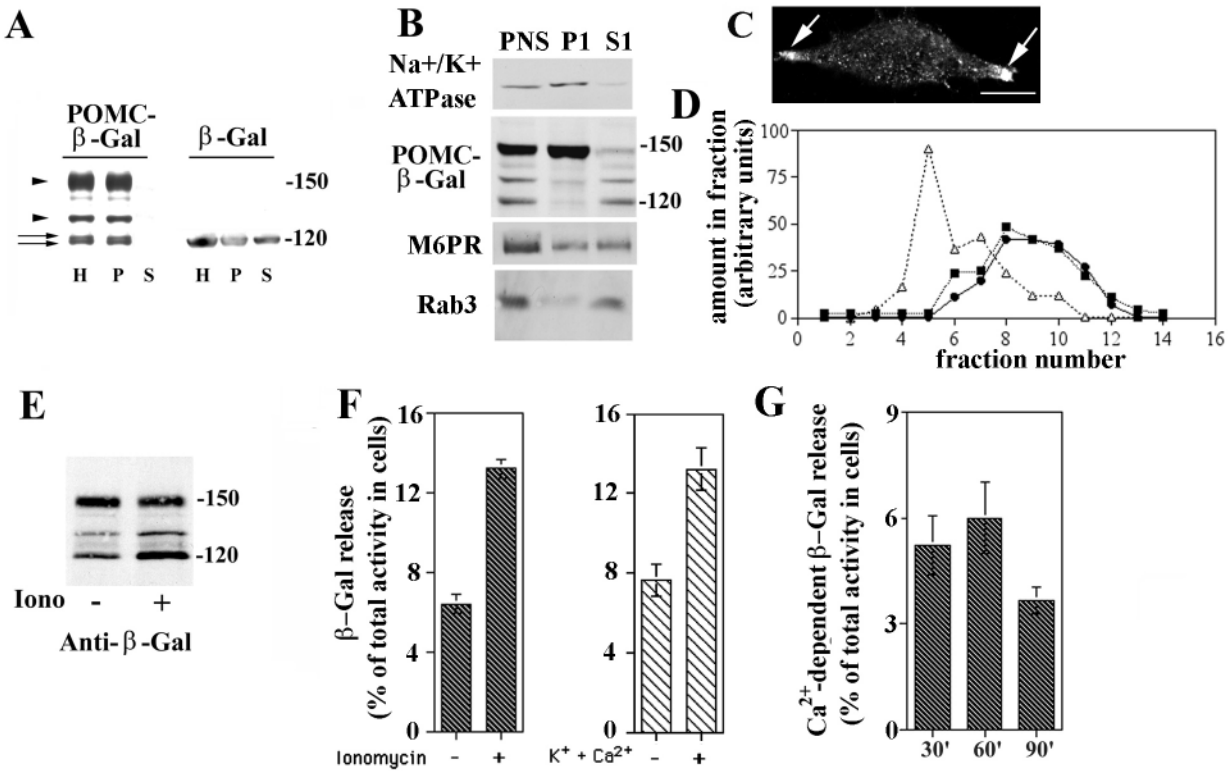

Fig. 1. POMC-β-Gal is processed and secreted in a Ca^{2+}-dependent manner. (A) Neuro2A cells expressing either POMC-β-Gal or β-Gal were homogenized (H) in homogenization buffer with protease inhibitors and centrifuged at 95,000 rpm for 30 minutes in a Beckman TLA 100.1 to obtain a membrane-containing pellet (P) or cytosol containing supernatant (S) as described previously (Koticha et al., 1999). Equal volumes (40 μl) of the samples were electrophoresed on 9% SDS-PAGE gels, transferred to nitrocellulose membranes and probed with anti-β-Gal antibodies. The arrowheads indicate bands that are detected with both the antibody against β-Gal and the antibody against ACTH. The upper arrow indicates the 124 kDa product of POMC-β-Gal cleavage. The lower arrow indicates β-Gal. (B) Post-nuclear supernatants (PNS) derived from N2A cells (0.4 ml) were centrifuged at 7,200 ***g*** for 10 minutes to obtain a pellet P1 and a supernatant S1. The pellet P1 was re-suspended in 0.4 ml of homogenization buffer. Equal volumes (40 μl) of the fractions were loaded onto an SDS-PAGE gels, transferred to nitrocellulose membranes and probed with anti-Na+/K+ ATPase, anti-β-Gal antibodies, anti-Mannose-6-Phophate receptor (M6PR) and anti Rab3 (monoclonal 42.1) antibodies. (C) Confocal immunofluorescence of cells stained with anti-Rab3 antibody 42.1. The arrows indicate Rab3 immunoreactivity at the tips. Bar, 10 μm. (D) The supernatant S1 was derived as in B was centrifuged at 70,000 ***g*** for 30 minutes, and the pellet was loaded onto a 20-60% (w/v) sucrose density gradient. The gradient was centrifuged at 50,000 rpm in the Beckman TLS-55 swinging bucket rotor. Fractions were collected from the top and analyzed by western blot with anti-mannose-6-phophate receptor antibodies (open triangles), anti-Rab3 antibodies (closed squares) and anti-β-Gal antibodies (closed circles, the 124/120 kDa band). (E) Neuro2A cells grown in 65 mm wells expressing POMC-β-Gal were pre-incubated at 37°C for 30 minutes in 1.0 ml M2 buffer with 0.7 mM $CaCl_2$. Cells were then incubated with 1 ml of M2 with or without 1 μM Ionomycin at 37°C for 1 hour. SDS-PAGE gel lanes were loaded with 50 μl of the cell medium. Western blot analysis of the secreted POMC-β-Gal products was performed using antibodies against β-Gal. Densitometry of the bands was done using the NIH Image 1.61software. This experiment was done three times. (F) Release of β-Gal activity (% age of total activity in cells) was measured from cells either incubated with Ca^{2+} alone (–) or treated with Ionomycin for 60 minutes (+) or depolarized by KCl for 120 minutes (+), as described in the Materials and Methods. The β-Gal activity release is an average of data from triplicate samples of a single experiment. This experiment was done four times with similar results. (G) The cells were incubated with Ca^{2+} alone (basal conditions) or with Ca^{2+} and Ionomycin (stimulated conditions) for 0-30, 0-60 and 0-90 minutes. Ca^{2+}-dependent β-Gal release (percentage of total activity in cells)=Rs_t-Rb_t, where Rs_t is the percentage of the total cell β-Gal activity that is released by samples stimulated with Ca^{2+} and Ionomycin, and Rb_t is the percentage of the total cell β-Gal activity that is released by samples kept in basal conditions. The average is calculated from the data of three independent experiments done with triplicate samples.

of stimulated to that of unstimulated cells is 0.96 (±0.12). Thus, the amounts of POMC-β-Gal precursor released by cells kept in basal and stimulated conditions are very similar. This observation is in agreement with the finding that unprocessed forms of POMC are secreted by the constitutive pathway (Gumbiner and Kelly, 1982). The ratio of the 120/124 kDa processed POMC-β-Gal products in the medium of stimulated to that of unstimulated cells is 4.36 (±0.73). Thus, processed POMC-β-Gal, unlike the 150 kDa precursor, is released into the medium in a regulated manner. The data are in agreement with the observation that in Neuro2A cells, Ca^{2+}-dependent release of β-endorphin hormone is four-fold more compared with that observed in basal conditions after 30 minutes incubation (Noel et al., 1989). In conclusion, the cell fractionation experiments and the secretion data support the concept that processed POMC-β-Gal is specifically targeted to granules that undergo regulated secretion.

We measured the amount of β-Gal activity released by Neuro2A cells transiently transfected with POMC-β-Gal. Cells treated for 1 hour in basal conditions released approximately 6% of their total β-Gal activity. Incubation with Ionomycin in the presence of Ca^{2+} lead to a two-fold increase in β-Gal secretion (Fig. 1F). Depolarizing the cells with 55 mM KCl in the presence of calcium for 2 hours lead to a similar increase in β-Gal release (Fig. 1F). The extent of Ca^{2+}-dependent release of β-Gal was similar at the 30 minute and 1 hour time points (Fig. 1G). Cells transfected with wild-type β-Gal released less than 0.5% of their total β-Gal activity when incubated for 1 hour in control conditions, whereas Ca^{2+}-dependent release of β-Gal activity was undetectable (data not shown). This observation indicates that, in the conditions used for our experiments, the fraction of damaged cells was minimal and that Ca^{2+}-dependent secretion depends on POMC-dependent targeting of β-Gal to the secretory pathway. By measuring β-Gal activity, it is not possible to discriminate between the unprocessed and the processed forms of POMC-β-Gal. The lower fold increase in regulated secretion (two-fold) measured by the β-Gal activity assay is probably because of the background of constitutively secreted POMC-β-Gal precursor (see Fig. 1E). In support of this possibility, the ratio of the sum of all three β-Gal-immunoreactive bands in the medium of stimulated to that of unstimulated cells was 1.86 (±0.23). Thus, the extent of total POMC-β-Gal release observed by using western blot analysis of the medium and the β-Gal activity assay is similar. Fig. 1E shows that only the processed forms of POMC-β-Gal undergo regulated secretion. Thus, it is likely that the Ca^{2+}-dependent secretion measured by the β-Gal activity assay is specifically caused by the release of the 137 and 124/120 POMC-β-Gal products. As the β-Gal activity assay is easier to quantify, we used this method to measure Ca^{2+}-dependent secretion in all the following experiments.

SNAP-25 is required for Ca^{2+}-dependent secretion in Neuro2A cells

Neuro2A cells have the SNARE components Syntaxin-1, VAMP-2, SNAP-25 and the non-neuronal SNAP-25 homologue Syndet/SNAP-23 (Fig. 2A). Endogenous SNAP-25 and exogenous myc-tagged SNAP-25A are prevalently localized at the plasma membrane (Fig. 2B,C). BoNT/E, a potent inhibitor of neurotransmission, cleaves SNAP-25 (Binz et al., 1994), and using BoNT/E toxin, SNAP-25 was shown to be specifically involved in Ca^{2+}-dependent secretion of hormones (Sadoul et al., 1997; Sadoul et al., 1995). To determine whether Ca^{2+}-dependent release of β-Gal activity occurs by the same pathway, we co-transfected BoNT/E-pcDNA3.1 and POMC-β-Gal in Neuro2A cells. We find that Ca^{2+}-stimulated secretion of β-Gal activity is almost abolished when BoNT/E light chain is expressed (Fig. 2D). When

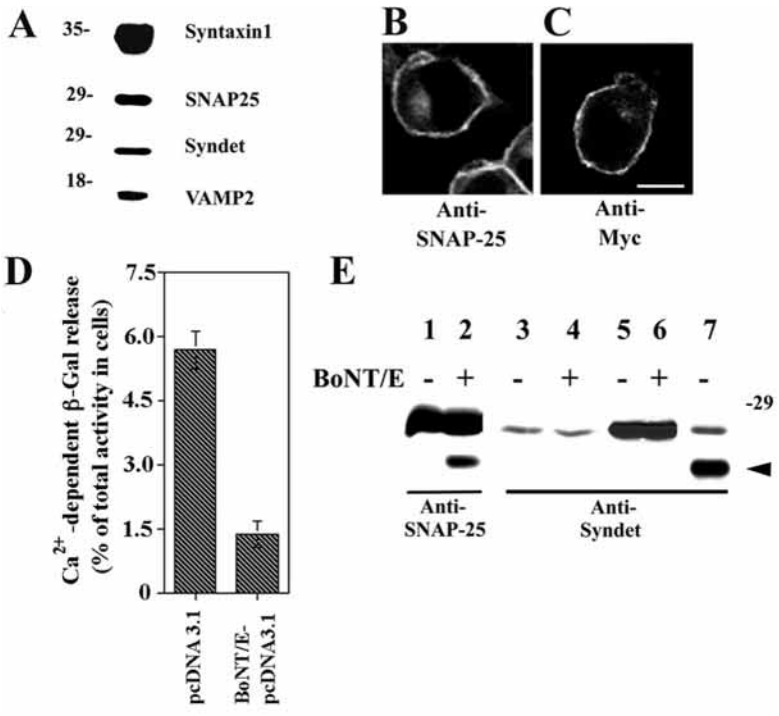

Fig. 2. SNAP-25 is required for Ca^{2+}-dependent secretion in Neuro2A cells. (A) Homogenates of Neuro2A cells were analyzed by western blot with antibodies against Syntaxin-1, SNAP-25, VAMP-2 and Syndet. (B) Confocal immunofluorescence image of wild-type Neuro2A cells stained with antibodies against SNAP-25. (C) Confocal immunofluorescence image of Neuro2A cells expressing myc-tagged SNAP-25A stained with antibodies against myc. Bar, 15 μm. (D) Neuro2A cells were transiently transfected with *POMC-β-Gal* and pcDNA3.1 or with *POMC-β-Gal* and *BoNT/E-pcDNA3.1*. Ca^{2+}-dependent β-Gal release is measured as described in Fig. 1G. The average is calculated from the data of four independent experiments done with triplicate samples. (E) Neuro2A cells were transiently transfected with *pcDNA3.1* (–) or *BoNT/E-pcDNA3.1* (+) alone (lanes 1-4) or in combination with *Syndet-pcB7* (lane 5 and 6) and *Syndet-Delta-BoNT/E-pcB7* (Koticha et al., 1999) (lane 7). Syndet-Delta-BoNT/E protein expressed in Neuro2A cells is a truncated protein that is identical to the expected product of Syndet digested by BoNT/E (arrowhead) (Washbourne et al., 2001). The western blot was probed with anti-SNAP-25 and anti-Syndet antibodies, as indicated.

BoNT/E was transiently expressed together with POMC-β-Gal in wild-type Neuro2A cells, approximately 10-15% of the endogenous SNAP-25 was cleaved (Fig. 2E, lanes 1 and 2). Since in these conditions the efficiency of cell transfection was 10%-15%, BoNT/E toxin cleaves most of the endogenous SNAP-25 protein in the transfected cells. We could not detect any cleavage of the endogenous or overexpressed Syndet/SNAP-23 by the toxin (Fig. 2E, lanes 3-6). These data are in agreement with the finding that Syndet/SNAP-23 is poorly digested by BoNT/E in vitro compared with SNAP-25 (Washbourne et al., 1997). These experiments show that SNAP-25 is required for regulated secretion of β-Gal activity and that Syndet/SNAP-23 is not involved in this process. Previously, it has been shown that SNAP-25 is necessary for hormone secretion and only highly overexpressed SNAP-23 (the human homologue of Syndet/SNAP-23) can replace SNAP-25 in regulated insulin release (Sadoul et al., 1997). Our results show that Ca^{2+}-stimulated secretion of β-Gal activity occurs by the same pathway as that of hormone release by endocrine cells.

The cysteine-rich domain of SNAP-25 is necessary to support regulated exocytosis in intact cells

To determine whether the cysteine-rich domain of SNAP-25A is important for function in intact cells, we generated a deletion mutant, Delta-SNAP-25A, that lacks the SNAP-25A sequence from ^{84}Cys to ^{95}Lys (Fig. 3A). We also generated a SNAP-25 mutant, CA-SNAP-25A, with its four cysteines changed to alanines (Fig. 3A). This mutant is the same as that used by Scales et al. to reconstitute regulated exocytosis in permeabilized PC-12 cells (Scales et al., 2000). In untransfected Neuro2A cells, more than 95% of the endogenous SNAP-25 is bound to membranes (data not shown). In transiently transfected cells, approximately 80% of foreign SNAP-25A is membrane bound (Fig. 3B). Thus, a minor fraction of SNAP-25 in the transfected cells is shifted into the cytosol, presumably because the membrane-binding machinery is saturated. In the same experiment, more than 95% of Delta-SNAP-25A is found in the soluble fraction, showing that deletion of the cysteine-rich domain shifts the protein into the cytosol. CA-SNAP-25A mutant is also found in the soluble fraction in transiently transfected Neuro2A cells (Fig. 3C). Thus, removing the cysteine-rich domain of SNAP-25 or substituting the cysteines with alanines makes the protein soluble in the transiently transfected Neuro2A cells. These data are in agreement with other reports (Lane and Liu, 1997; Veit et al., 1996).

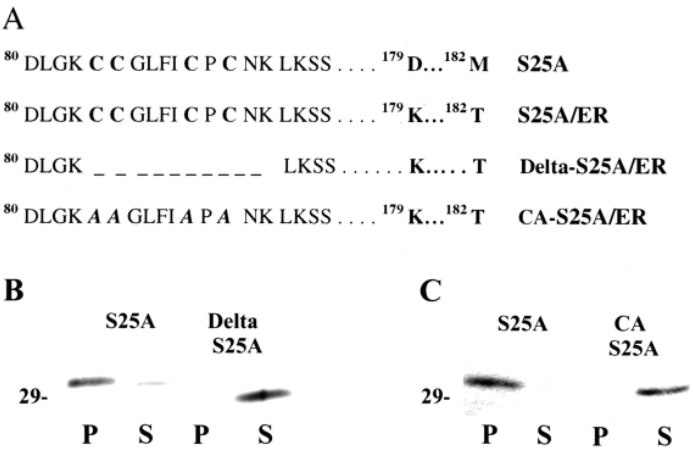

Fig. 3. The cysteine-rich domain of SNAP-25 is necessary for membrane binding. (A) The SNAP-25A mutants used in this study. BonT/E-resistant SNAP-25 SNAP-25A/ER (S25A/ER) has amino acids ^{179}Asp and ^{182}Met changed to ^{179}Lys and ^{182}Thr. BonT/E-resistant Delta-SNAP-25A/ER (Delta-S25A/ER) lacks 11 amino acids corresponding to the cysteine-rich domain of SNAP-25A and has the corresponding Asp and Met residues changed to Lys and Thr. BonT/E-resistant CA-SNAP-25A/ER (CA-S25A/ER) has all cysteines substituted with alanines and has amino acids ^{179}Asp and ^{182}Met changed to ^{179}Lys and ^{182}Thr. (B) Fractionation of cells transiently expressing wild-type SNAP-25A or Delta-SNAP-25A. Post-nuclear supernatants from these cells were centrifuged as described in Fig. 1 and separated into membrane pellet (P) or cytosol (S). Equal volumes of the fractions were then loaded onto a 13% SDS-PAGE gel, transferred and probed with anti-Myc antibody. (C) Fractionation of cells transiently expressing either wild-type SNAP-25A or CA-SNAP-25A. This experiment was done as described in panel B.

Unlike SNAP-25, SNAP-23 is resistant to proteolysis by BoNT/E (Sadoul et al., 1997; Washbourne et al., 2001). To obtain a SNAP-25 protein resistant to BoNT/E digestion, we mutated its cleavage site sequence to that of SNAP-23. SNAP-25A residue ^{179}Asp was mutated into a Lys, and residue ^{182}Met was mutated into a Thr (Fig. 3A) to obtain the toxin-resistant construct SNAP-25A/ER-pcB7. This SNAP-25A/ER-pcB7 construct was mutated to obtain the Delta-SNAP-25A/ER-pcB7 and the CA-SNAP-25/ER-pcB7 constructs. All of these constructs have the myc-epitope tag at the C-terminus.

We generated a Neuro2A cell line, G14, that stably expresses BoNT/E toxin. In the G14 cells, all of the endogenous SNAP-25A is cut by the toxin. The cleaved SNAP-25 thus migrates faster than the intact SNAP-25 protein (Fig. 4A, compare lane 1 with lane 2). We transiently transfected the SNAP-25A/ER-pcB7 and Delta-SNAP-25A/ER-pcB7 plasmids in G14 cells. Both SNAP-25A/ER and Delta-SNAP-25A/ER proteins have the myc-tag at the C-terminus and, thus, migrate with higher molecular weight than endogenous SNAP-25 (Fig. 4A, compare lane 1 with lanes 3 and 4). If cut by the toxin, the myc-tagged SNAP-25A/ER protein would generate a peptide with the same size as the endogenous BoNT/E SNAP-25 fragment, whereas myc-tagged Delta-SNAP-25A/ER would generate a smaller peptide. We find that the size and the amount of the cleaved SNAP-25 fragment is the same in G14 cells expressing BoNT/E and transfected with POMC-β-Gal together with pcB7, SNAP-25A/ER-pcB7 or Delta-SNAP-25A/ER-pcB7 (Fig. 4A, compare lane 2 with lanes 3 and 4). We conclude that SNAP-25A/ER and Delta-SNAP-25A/ER proteins are resistant to BoNT/E and that expression of these proteins does not inhibit the cleavage of endogenous SNAP-25A by BoNT/E. These experiments also show that similar amounts of SNAP-25A/ER and Delta-SNAP-25A/ER are expressed in G14 cells. We obtained the same results when the G14 cells were transfected with the CA-SNAP-25A/ER construct (data not shown). We estimated that the amount of CA-SNAP-25A/ER expressed is approximately 0.025 pg per transfected cell (see Materials and Methods), leading to a cytosolic concentration of approximately 1 μM. The western blot in Fig. 4A shows that the amount of endogenous, cleaved

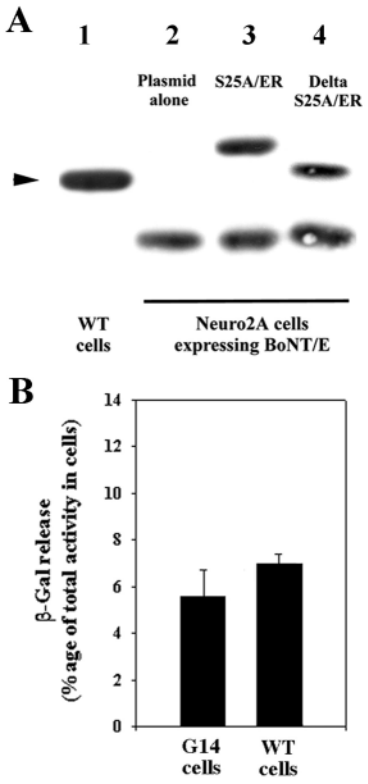

Fig. 4. Expression of BoNT/E-resistant SNAP-25A mutants does not inhibit the cleavage of endogenous SNAP-25 by the toxin. (A) Homogenates of wild-type Neuro2A cells (lane 1) and of cells stably expressing BoNT/E, the G14 cells (lane 2-4). G14 cells were transiently transfected with *POMC-β-Gal* together with either pcB7 (lane 2) or *SNAP-25A/ER-pcB7* (lane 3) or *Delta-SNAP-25A/ER-pcB7* (lane 4). Homogenates of cells expressing the indicated constructs were electrophoresed on 13% SDS-PAGE gels, and western blots were probed with anti-SNAP-25 antibody. The arrowhead indicates the endogenous SNAP-25. (B) Release of β-Gal activity under basal conditions from G14 cells and wild-type Neuro2A cells transfected with *POMC-β-Gal* construct. The release is calculated as a percentage of the total β-Gal activity in the cells.

SNAP-25 in the G14 cells is similar to that of exogenous SNAP-25. Co-transfection of the plasmids POMC-β-Gal together with pcB7, SNAP-25A/ER-pcB7 or Delta-SNAP-25A/ER-pcB7 in G14 cells results in a transfection efficiency of approximately 25%. As the exogenous SNAP-25 is expressed only in 25% of the cell population, we deduce that the amount of endogenous SNAP-25 corresponds to one fourth of the exogenous protein and is 0.00625 pg per cell or approximately 150,000 molecules per cell. G14 cells have similar morphology and generation time to the Neuro2A cells (data not shown). Unstimulated G14 cells release the same amount of β-Gal activity as wild-type Neuro2A (Fig. 4B) cells, indicating that the release of β-Gal activity observed in basal conditions is not affected by BoNT/E expression.

A full-length SNAP-25 protein with its cysteines mutated to alanines is able to rescue regulated secretion in permeabilized, BoNT/E-treated PC12 cells (Scales et al., 2000). We expressed the same level of full-length, soluble SNAP-25 (SNAP-25A/ER) and wild-type SNAP-25 to study the role of the cysteine-rich domain in intact cells. Regulated exocytosis of β-Gal activity was measured in cells lacking endogenous SNAP-25. G14 cells stably expressing BoNT/E were transiently transfected with *POMC-β-Gal* in combination with pcB7 or SNAP-25A/ER or Delta-SNAP-25A/ER or CA-SNAP-25A/ER (Fig. 5A,B). The variability between the average β-Gal activities of each of these groups of cells was less than 10%. This observation indicates that expression of different SNAP-25 proteins did not change the level of expression of POMC-β-Gal. Cells co-tranfected with POMC-β-Gal and the pcB7 vector (Fig. 5A,B) or with POMC-β-Gal alone (data not shown) did not release β-Gal activity in response to increase in intracellular Ca^{2+}. These data further support the concept that regulated release of β-Gal activity requires SNAP-25 (Fig. 2A). In agreement with this concept, Ca^{2+}-stimulated release of β-Gal-activity is reconstituted in G14 cells expressing SNAP-25A/ER (Fig. 5A,B). In these cells, the extent of Ca^{2+}-dependent β-Gal release was approximately 65% of that of wild-type Neuro2A cells. It is possible that SNAP-25A/ER with mutations at ^{179}Asp and at ^{182}Met and the Myc epitope tag is less efficient than wild-type SNAP-25 in reconstituting regulated secretion. Another possibility is that expression of SNAP-25A/ER does not occur in all the cells expressing the POMC-β-Gal protein, thus lowering the efficiency by which regulated release is reconstituted.

Hormone secretion is specifically dependent on intact SNAP-25. In this assay, β-Gal activity release is abolished by BoNT/E toxin digestion of SNAP-25 and reconstituted by

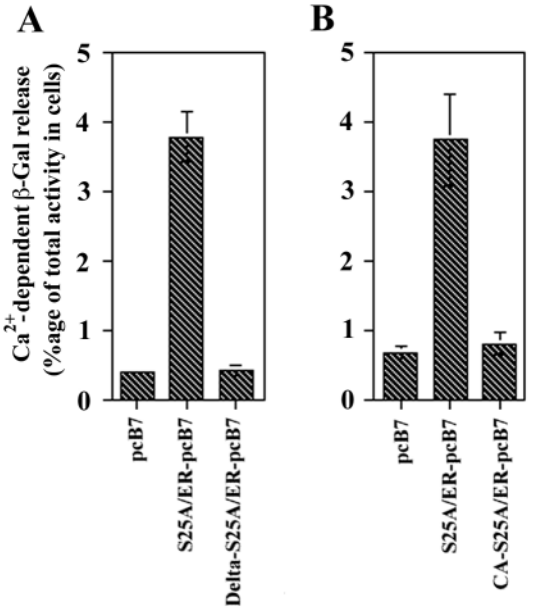

Fig. 5. The cysteine-rich domain of SNAP-25 is necessary to support regulated exocytosis in intact cells. (A) Ca^{2+}-dependent secretion of β-Gal activity in G14 cells stably expressing BoNT/E and transiently transfected with *POMC-β-Gal* and pcB7 or *SNAP-25A/ER-pcB7* or *Delta-SNAP-25A/ER-pcB7*. (B) Ca^{2+}-dependent secretion of β-Gal activity in G14 cells stably expressing BoNT/E and transiently transfected with *POMC-β-Gal* and pcB7 or *SNAP-25A/ER-pcB7* or *CA-SNAP-25A/ER-pcB7*. Ca^{2+}-dependent β-Gal release (as in Fig. 1G) is calculated from the data of four independent experiments done with triplicate samples.

expression of toxin-resistant SNAP-25. These data further confirms the conclusion that Ca^{2+}-dependent β-Gal activity release occurs by the same pathway as native hormone release (Fig. 2). The amount of Ca^{2+}-dependent β-Gal released from cells expressing either Delta-SNAP-25A/ER (Fig. 5A) or CA-SNAP-25A/ER (Fig. 5B) was equal to that released by G14 cells transfected with control plasmid. Thus, soluble SNAP-25 mutant proteins are unable to reconstitute Ca^{2+}-dependent secretion of β-Gal activity when expressed at the same level as the palmitoylated protein. We conclude that the cysteine-rich domain of SNAP-25 is necessary for function in intact cells.

The cysteine-rich domain of SNAP-25 is important for SNARE complex formation in intact cells

Homogenates derived from G14 cells, transiently co-transfected with *POMC-β-Gal* and *SNAP-25A/ER*, unlike those derived from cells co-transfected with pcB7 or *CA-SNAP-25A/ER* (Fig. 6A, arrowhead) or *Delta-SNAP-25A/ER* (Fig. 6D, arrowhead) have a prominent SDS-resistant 99 kDa band that is detected by the anti-SNAP-25 antibody. The 99 kDa band is the SNARE complex because it dissociates after boiling to yield Myc-SNAP-25, Syntaxin-1 and VAMP-2 proteins (data not shown). The 99 kDa band is also detected by anti-Myc antibodies in homogenates derived from G14 cells transfected with *SNAP-25A/ER* but not in the same samples derived from cells transfected with pcB7 or *CA-SNAP-25A/ER* (Fig. 6B). This observation indicates that only exogenous SNAP-25A/ER, but not CA-SNAP-25A/ER or Delta-SNAP-25A/ER, is able to form complexes in the conditions used for this experiment. The fainter, lower, SNAP-25-reactive bands that are detected in G14 cells transfected with control plasmid, or in G14 cells expressing CA-SNAP-25A/ER (Fig. 6A, double arrows) or Delta-SNAP-25A/ER (Fig. 6D, double arrows), may correspond to SNAP-25 complexes formed by the endogenous BoNT/E-cut SNAP-25. SNARE complex formation is inhibited by cleavage of SNAP-25 (Hayashi et al., 1994); thus, it is possible that the faster migrating bands correspond to other SNAP-25 complexes, perhaps disulfide-linked SNAP-25 oligomers (Sadoul et al., 1997a). The total amount of exogenous wild-type and soluble SNAP-25 proteins expressed in G14 cells was comparable (Fig. 6C,E). These data indicate that soluble SNAP-25 proteins cannot form SNARE complexes when expressed to the same level as the exogenous membrane-bound protein. The cysteine-rich domain is not involved in SNARE complex formation (Fasshauer et al., 1998; Poirier et al., 1998). The soluble mutant may not form complexes in the Neuro2A cells because, unlike the wild-type protein, it is not concentrated near the plasma membrane. To test this possibility, we expressed CA-SNAP-25A/ER at five-fold higher levels in G14 cells (Fig. 6F, lower panel). In cells transfected with 10 μg of *CA-SNAP-25A/ER-pcB7* plasmid, the soluble SNAP-25 is expressed at sufficient levels to support SNARE complex formation (Fig. 6F, upper panel, arrowhead), as indicated by the appearance of a slower migrating band above the endogenous SNAP-25 complexes (Fig. 6F, upper panel, double arrows). We conclude that the cysteine-rich domain of SNAP-25, by targeting SNAP-25 to the plasma membrane, is important for increasing its local concentration and thereby supporting SNARE complex formation.

A truncated SNAP-25A protein having amino acids 82-206 rescues regulated exocytosis

It has been proposed that a ternary SNARE complex could be formed by the C- and the N-terminal domains of two distinct SNAP-25 molecules (Fasshauer et al., 1998). The flexible linker region connecting the two helices of SNAP-25 is necessary for multimerization of SNARE complexes (Fasshauer et al., 1998; Poirier et al., 1998). The possible significance of SNARE complex multimerization in regulated exocytosis is not clear, as the SNAP-25 peptides corresponding to the C-terminus and the N-terminus helices not linked by the loop were able to rescue exocytosis in the in vitro assays (Chen et al., 1999; Parlati et al., 1999). These data suggested that if

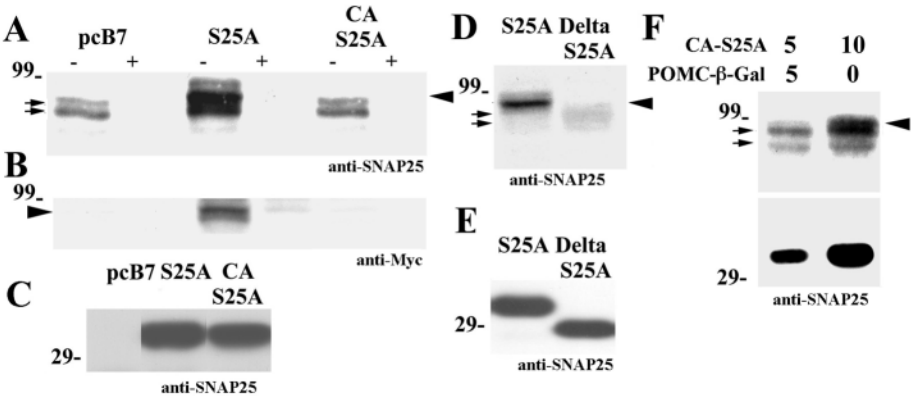

Fig. 6. The cysteine-rich domain of SNAP-25 is important for SNARE complex formation in intact cells. (A) Post-nuclear supernatants in sample buffer were prepared as described in the Materials and Methods. The samples were either not boiled (–) or boiled (+)and loaded on a 9% SDS-PAGE gel. SNAP-25 complexes from G14 cells transiently transfected with 5 μg of *POMC-β-Gal* together with 5 μg of pcB7 or *SNAP-25A/ER-pcB7* (S25A) or *CA-SNAP-25A/ER-pcB7* (CA S25A) were detected by western blot analysis with anti-SNAP-25 antibody. The arrowhead indicates SNARE complexes formed by exogenous SNAP-25 and the double arrow indicated SNAP-25 complexes formed by endogenous SNAP-25. (B) The western blot of a similar experiment was probed with anti-Myc antibody. (C) The total amount of SNAP-25 protein expressed in the transfected cells shown in A is detected with the anti-SNAP25 antibody. (D) SNAP-25 complexes from G14 cells transiently transfected with *POMC-β-Gal* together with either *SNAP-25A/ER-pcB7* or *Delta-SNAP-25A/ER-pcB7* were detected as described in A. (E) The total amount of SNAP-25 monomer expressed in these cells was detected as in C. These experiments were repeated three times with triplicate samples per experiment. (F) SNAP-25 complexes from G14 cells transiently transfected with the indicated amounts (μg) of *POMC-β-Gal* and *CA-SNAP-25A/ER-pcB7* or *CA-SNAP-25A/ER-pcB7* alone. The arrowhead indicates SNARE complexes formed by exogenous SNAP-25 and the double arrow indicates complexes formed by endogenous SNAP-25. The total amount of CA-SNAP-25A/ER expressed in the cells is shown in the lower panel. The western blot was probed with the anti-SNAP-25 antibody.

the SNAP-25 C-terminus helix is efficiently targeted to the plasma membrane it should rescue regulated secretion in the G14 cells expressing BoNT/E. To test this possibility, we expressed the SNAP-25 C-terminus attached to the loop with the entire cysteine-rich domain, producing S25A/ER-82-206 (Fig. 7A). The minimal plasma membrane-targeting domain of SNAP-25 maps to residues 85-120 (Gonzalo et al., 1999); thus, it is expected that a SNAP-25 mutant lacking the entire N-terminus domain would still be targeted to the plasma membrane. Fig. 7B shows that S25A/ER-82-206 protein is efficiently targeted to the plasma membrane. When S25A/ER-82-206 protein was transiently transfected in G14 cells, it was able to rescue Ca^{2+}-dependent secretion of β-Gal activity as efficiently as the wild-type SNAP-25 protein (Fig. 5). The S25A/ER-82-206 protein was also able to form SNARE complexes of 70 kDa (Fig. 7D), which are similar to the complexes formed by the C-terminal helix of SNAP-25 introduced in permeabilized PC12 cells (Chen et al., 1999). The C-terminal helix of SNAP-25, without the membrane-binding region, S25A-ER-140-206, is highly unstable when expressed in cells, and its activity cannot be estimated. These experiments show that the two helices of SNAP-25 do not need to be physically linked in order to function in intact cells. As SNAP-25 residues 20-80 are required for SNAP-25 binding to syntaxin, these experiments support the concept that that the interaction between SNAP-25 and sytaxin is not necessary for membrane binding and plasma-membrane targeting.

Discussion

In neuroblastoma cells, a chimera with β-Gal fused to the C-terminus of POMC is targeted to a specialized secretory pathway. This conclusion is supported by the following observations. First, POMC is processed, whereas the β-Gal tag remains intact. As hormone processing occurs only in secretory granules (Orci et al., 1987; Tooze et al., 1987) endoproteolytic cleavage of the tagged pro-hormone indicates its correct sorting to these vesicles. Second, processed POMC-β-Gal, unlike the precursor, co-fractionates with Rab3 proteins that are associated with dense core granules. Third, precursor POMC-β-Gal is released constitutively, whereas processed POMC-β-Gal is released in a Ca^{2+}-dependent manner. Similarly, the release of approximately 5% of the total β-Gal activity in the medium occurs in a regulated manner, as it is induced by depolarization and is Ca^{2+}-dependent. Fourth, the fraction of β-Gal activity released in a Ca^{2+}-dependent manner is abolished by BoNT/E expression, whereas secretion in cells kept under control conditions is unchanged. Fifth, expression of a BoNT/E-resistant SNAP-25 protein rescues Ca^{2+}-dependent β-Gal secretion. As exocytosis of native hormones specifically requires intact SNAP-25 (Sadoul et al., 1997; Sadoul et al., 1995), these data indicate that Ca^{2+}-dependent secretion of β-Gal activity occurs by the same pathway.

A number of studies have demonstrated that SNAP-25 and Syndet/SNAP-23 are targeted to the plasma membrane by the cysteine-rich domain (Gonzalo et al., 1999; Gonzalo and Linder, 1998a; Koticha et al., 1999; Veit et al., 2000; Vogel and Roche, 1999). However, the biological significance of the membrane localization of endogenous SNAP-25 remains under discussion, as one study found that soluble SNAP-25 proteins functions in regulated exocytosis in HIT cells (Gonelle-Gispert et al., 2000), whereas a more recent paper shows that soluble SNAP-25 is inactive in PC12 cells (Washbourne et al., 2001). A discrepancy in the results may be caused by different levels of overexpressed SNAP-25 in the transfected cells. It is also possible that the permeabilized cell system used in both studies leads to depletion of different amounts of soluble SNAP-25 proteins through the pores created by the toxin at the plasma membrane (Gonelle-Gispert et al., 2000; Washbourne et al., 2001). To resolve the question of whether membrane localization is required for SNAP-25 function in the cell, we measured secretion from intact Neuro2A cell lines stably transfected with BoNT/E toxin.

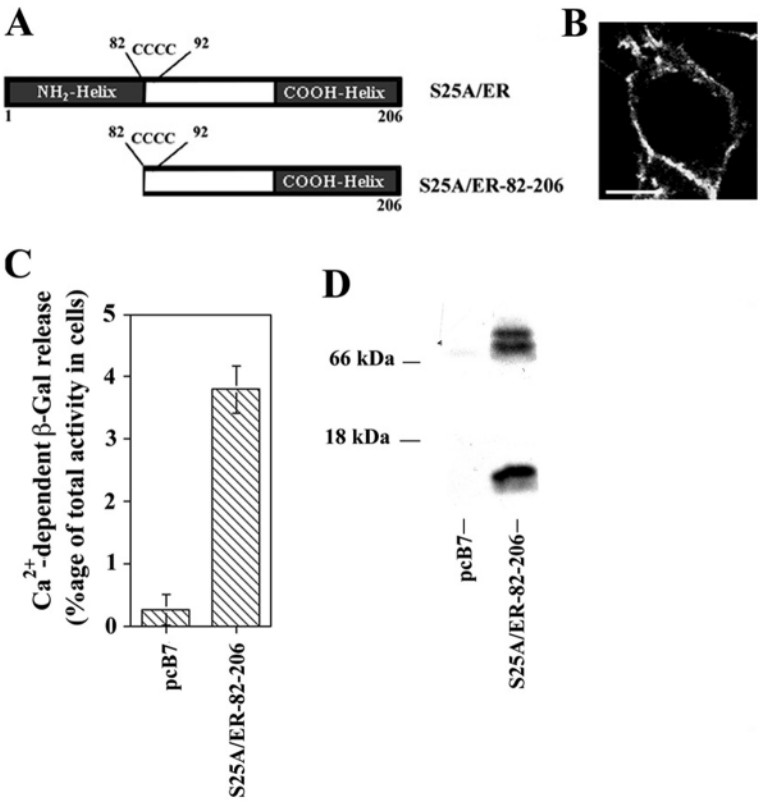

Fig. 7. The two helices of SNAP-25 can function independently of each other in regulated exocytosis. (A) A SNAP-25 construct called *S25A/ER-82-206-pcB7*, lacking the N-terminal helical region and containing amino acids 82-206 was generated. (B) Confocal immunofluorescence image of Neuro2A cells expressing myc-tagged S25A/ER-82-206 stained with antibodies against myc. Bar, 15 μm. (C) Ca^{2+}-dependent secretion of β-Gal activity (percentage of total activity in cells) was derived as in Fig. 5 A. β-Gal secretion is measured in G14 cells stably expressing BoNT/E and transiently transfected with *POMC-β-Gal* and *pcB7* or *S25A/ER-82-20-pcB7*. Ca^{2+}-dependent release of β-Gal (as in Fig. 1G) is calculated from the data of four independent experiments done with triplicate samples as in Fig. 1. (D) SNARE complexes derived from G14 cells transiently transfected with *POMC-β-Gal* together with either *pcB7* or *S25A/ER-82-206-pcB7* were detected as described in Fig. 6. The total amount of S25A/ER-82-206 expressed is shown in the lower panel probed with anti-myc antibodies.

Transfected neuroblastoma cells are dependent on expression of exogenous, BoNT/E-resistant SNAP-25 for regulated secretion. By using the Neuro2A cells, it was possible to achieve a level of exogenous SNAP-25 overexpression in the transiently transfected cells that was only four-fold higher than endogenous SNAP-25. Moreover, the effect of membrane-bound and soluble SNAP-25 could be directly compared as no permeabilization procedures are used prior to stimulation of the cells. Under these conditions we find that soluble, BoNT/E-resistant SNAP-25 mutants, either lacking the cysteine-rich domain or with the four cysteines residues changed to alanines, are unable to support exocytosis.

In vitro reconstitution of norepinephrine secretion occurs at concentrations of 10-40 μM of full-length, soluble SNAP-25 with its cysteine residues mutated into alanines (Scales et al., 2000). In the Neuro2A cells used here, the intracellular concentration of the same soluble mutated SNAP-25 protein is 1 μM, which is below the level required to reconstitute granule release in vitro (Chen et al., 1999; Scales et al., 2000). Targeting of SNAP-25 to the plasma membrane or to cholesterol-dependent clusters at which secretory vesicles preferentially dock and fuse (Lang et al., 2001) would greatly increase the concentration of SNAP-25 to the levels required for exocytosis. We conclude that the role of the cysteine-rich domain is to increase the concentration of SNAP-25 near the plasma membrane to a sufficient level to allow exocytosis.

Mutation of the palmitoylation site of G proteins or the GRK class of serine/threonine kinases impairs their function in signal transduction [reviewed by Resh (Resh, 1999)]. Similarly, it is possible that palmitoylation of SNAP-25 modulates its activity by controlling its cell localization. It has been proposed that SNAP-25 is a major substrate for palmitoylation in adult CNS and that palmitoylation is necessary to target newly synthesized SNAP-25 to the plasma membrane (Gonzalo et al., 1999; Gonzalo and Linder, 1998a; Hess et al., 1992). Our results show that mutants at the palmitoylation site of SNAP-25 are unable to support regulated exocytosis. Together, these findings suggest that inhibition of SNAP-25 palmitoylation lead to a pool of newly synthesized SNAP-25 that is inactive. Mature SNAP-25 is dynamically palmitoylated in PC12 cells and in neurites (Hess et al., 1993; Lane and Liu, 1997); thus, in principle, it is possible that the activity of mature, processed SNAP-25 is controlled by cycles of pamitoylation and depalmitoylation. Against this possibility, it has been shown that chemical deacylation of SNAP-25 does not release the protein from its membrane association (Gonzalo and Linder, 1998a), possibly because of its interaction with syntaxin (Gonelle-Gispert et al., 2000; Vogel et al., 2000; Washbourne et al., 2001).

SNARE complexes can be formed in vitro by soluble Syntaxin-1, VAMP-2 and the SNAP-25 N- and C-terminal fragments. Thus, the loop that connects the N- and C-terminal coils of SNAP-25, including the cysteine-rich motif, does not participate in complex formation in vitro (Fasshauer et al., 1998; Poirier et al., 1998; Sutton et al., 1998). Here, we find that a SNAP-25 mutant, either lacking the cysteine-rich domain or with its cysteines substituted with alanines, does not form SNARE complexes when expressed at the same level as wild-type SNAP-25 in transfected neuroblastoma cells. To observe SNARE complexes, exogenous soluble SNAP-25 protein was expressed at approximately five-fold higher levels than the exogenous membrane-bound SNAP-25. The only known role of the cysteine-rich domain is to target SNAP-25 to the plasma membrane. Thus, the simplest interpretation of these results is that the cysteine-rich domain, by controlling the localization of SNAP-25 at the plasma membrane, regulates its availability to form SNARE complexes in the cell.

We have also shown that the C-terminal and the N-terminal helices of SNAP-25, each targeted to the plasma membrane by two distinct cysteine-rich domains, function as well as full-length SNAP-25 in intact cells. These results are in agreement with the in vitro finding that the two helices do not need to be linked together to function (Chen et al., 1999; Parlati et al., 1999; Scales et al., 2000). Although the loop may act as a linker of multiple SNARE complexes, this function does not appear to be necessary for function in the cell.

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