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Complexin 2 Modulates Vesicle-associated Membrane Protein (VAMP) 2-regulated Zymogen Granule Exocytosis in Pancreatic Acini*[S]

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Michelle A. Falkowski, Diana D. H. Thomas, and Guy E. Groblewski[1]

From the Department of Nutritional Sciences, University of Wisconsin, Madison, Wisconsin 53706

Complexins are soluble proteins that regulate the activity of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes necessary for vesicle fusion. Neuronal specific complexin 1 has inhibitory and stimulatory effects on exocytosis by clamping *trans*-SNARE complexes in a prefusion state and promoting conformational changes to facilitate membrane fusion following cell stimulation. Complexins are unable to bind to monomeric SNARE proteins but bind with high affinity to ternary SNARE complexes and with lower affinity to target SNARE complexes. Far less is understood about complexin function outside the nervous system. Pancreatic acini express the complexin 2 isoform by RT-PCR and immunoblotting. Immunofluorescence microscopy revealed complexin 2 localized along the apical plasma membrane consistent with a role in secretion. Accordingly, complexin 2 was found to interact with vesicle-associated membrane protein (VAMP) 2, syntaxins 3 and 4, but not with VAMP 8 or syntaxin 2. Introduction of recombinant complexin 2 into permeabilized acini inhibited Ca^{2+}-stimulated secretion in a concentration-dependent manner with a maximal inhibition of nearly 50%. Mutations of the central α-helical domain reduced complexin 2 SNARE binding and concurrently abolished its inhibitory activity. Surprisingly, mutation of arginine 59 to histidine within the central α-helical domain did not alter SNARE binding and moreover, augmented Ca^{2+}-stimulated secretion by 130% of control. Consistent with biochemical studies, complexin 2 colocalized with VAMP 2 along the apical plasma membrane following cholecystokinin-8 stimulation. These data demonstrate a functional role for complexin 2 outside the nervous system and indicate that it participates in the Ca^{2+}-sensitive regulatory pathway for zymogen granule exocytosis.

Complexin 1, originally identified as synaphin, is a small cytosolic protein that associates with soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes, which are essential to drive membrane fusion during neurotransmitter exocytosis (1, 2). Although complexin 1 is expressed exclusively in neurons, complexin 2 appears to be ubiquitously expressed and presumably functions in other secretory cell systems. Complexins 1 and 2 are highly homologous, each containing 134 amino acids that are 86% identical (3). Moreover, rat, mouse, and human complexin 2 proteins are 100% identical, although the nucleotide sequences of their mRNAs are considerably different (4). Recently, two other isoforms, complexins 3 and 4, were identified; however, evidence of their specific function is unclear. Complexin 3 is expressed strongly in retina and certain regions of the brain including the hippocampus and thalamus, whereas complexin 4 is only expressed in retina (5).

In neurons, the SNARE complex is a four-helical bundle composed of vesicle associated membrane protein (VAMP)[2] 2, and the target SNAREs (t-SNAREs), syntaxin 1 and SNAP 25, located on the plasma membrane (6). Complexin 1 was originally thought to clamp SNARE complexes at a prefusion step, thereby inhibiting exocytosis (7, 8). Upon elevation of intracellular Ca^{2+}, complexin 1 was proposed to be displaced from the SNARE complex by synaptotagmin 1, in turn allowing the final stages of neurotransmitter release to commence (7). More recent studies have shown that in addition to this clamping activity, complexin 1 also directly facilitates the final stages of exocytosis (9, 10).

Complexins comprise an N terminus (amino acids 1–29), accessory α-helix (amino acids 30–48), central α-helix (amino acids 49–70), and a C terminus (amino acids 71–134) (see Fig. 4). Complexin 1 is unable to bind to monomeric SNARE proteins but binds with high affinity to the ternary SNARE complex (4, 10–13) and with lower affinity to the t-SNARE complex containing syntaxin 1 and SNAP 25 (4, 10). The central α-helix of complexin 1 binds the ternary SNARE complex as an antiparallel helix (14–18) in the groove between the VAMP 2 and syntaxin helices (14). Xue *et al.* conducted a detailed mutational analysis of complexin 1 SNARE binding and secretory function demonstrating that although the central α-helix is essential for SNARE binding, the adjacent accessory α-helix has a negative effect, and the N terminus a positive effect on exocytosis (17). Maximov *et al.* reported more recently that complexin 1 functions in neurons by simultaneously suppressing spontaneous vesicle fusion and activating fast Ca^{2+}-stimulated fusion. Further evidence was provided to support that complexin 1 binding controls the force that *trans*-SNARE complexes apply onto opposing membranes to facilitate their fusion (18).

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1 and Movies S1–S3.

[1] To whom correspondence should be addressed: Dept. of Nutritional Sciences, University of Wisconsin, 1415 Linden Dr., Madison, WI 53706. Tel.: 608-262-0884; Fax: 608-262-5860; E-mail: groby@nutrisci.wisc.edu.

[2] The abbreviations used are: VAMP, vesicle-associated membrane protein; CCK, cholecystokinin; PFO, perfringolysin O; t-SNARE, target SNARE; ZG, zymogen granule; ZGM, zymogen granule membrane.

The majority of work conducted thus far has utilized complexin 1 in *in vitro* liposome fusion assays or neuronal systems. Studies on complexin 2 in neural, chromaffin, and mast cells indicate that this isoform also plays a functional role in Ca^{2+}-triggered exocytosis (9, 19–21). However, comparably less is understood about complexin 2 in secretory cells which express isoforms of the neuronal SNAREs including SNAP 23 and various VAMPs, syntaxins, synaptotagmins, and/or synaptotagmin-like proteins. Tadokoro *et al.* demonstrated that knockdown of complexin 2 expression in RBL-2H3 mast cells attenuated Ca^{2+}-dependent degranulation (9), and GST-complexin 2 was shown to interact with SNAP 23, syntaxin 3, VAMP 2, and to a lesser extent VAMP 8 in cell lysates (19).

Pancreatic acinar cells are a prototypical model of exocrine secretory cells that respond to elevated intracellular Ca^{2+} by stimulating exocytosis of digestive enzymes necessary for the assimilation of the diet. These cells contain at least two populations of zymogen secretory granules (ZGs) based on their expressions of VAMP 2 or 8 (22). Further, both VAMP 2 and VAMP 8 differentially modulate Ca^{2+}-stimulated secretion with VAMP 2-containing ZGs mediating the earliest stages and VAMP 8 the later stages of the secretory response. In the present study we examined a potential role for complexin 2 in modulating digestive enzyme secretion in acinar cells. Results indicate that complexin 2 is concentrated along the apical plasma membrane and interacts with SNARE complexes containing VAMP 2 and syntaxins 3 and 4. Use of site-specific and truncation mutants of complexin 2 regulatory domains in permeabilized cells indicates that the molecule has both stimulatory and inhibitory effects on Ca^{2+}-stimulated secretion, providing molecular evidence of complexin 2 function outside of the nervous system.

EXPERIMENTAL PROCEDURES

Antibodies—Two polyclonal anti-complexin 1,2 (catalog numbers 122 002 and 122 102), monoclonal anti-VAMP 2 (catalog number 104 211), and polyclonal anti-syntaxin 2, 3, and 4 (catalog numbers 110 123, 110 033, and 110 042, respectively) were purchased from Synaptic Systems. A polyclonal antibody to the cytosolic domain of recombinant human VAMP 8 was produced in rabbits (23). Polyclonal anti-SNAP 23 (catalog number ab79180) was purchased from Abcam.

Other Reagents—Soybean trypsin inhibitor, benzamidine, phenylmethanesulfonyl fluoride (PMSF), HEPES, goat serum, cold-water fish gelatin, and Triton X-100 were purchased from Sigma-Aldrich; essential amino acid solution from GIBCO; and a protease inhibitor mixture containing AEBSF, aprotinin, EDTA, leupeptin, and E64 from Calbiochem. A Phadebas amylase assay kit, chloroform, and isopropyl alcohol were purchased from Fisher Scientific. Alexa Fluor 488- and 546-conjugated rabbit and mouse secondary antibodies, rhodamine, and Alexa Fluor-647 conjugated phalloidin, ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPI) and TRIzol were purchased from Invitrogen. TissueTek O.C.T. compound was purchased from Sakura Finetek. Protein determination reagent was purchased from Bio-Rad. Peroxidase-conjugated sheep anti-mouse IgG and donkey anti-rabbit IgG and glutathione-Sepharose high performance beads were from GE Healthcare. Easy-A Hi-Fi PCR cloning enzyme was purchased from Stratagene. Disuccinimidyl suberate was purchased from Thermo Scientific. The perfringolysin O (PFO) bacterial expression plasmid was a kind gift from A. Johnson and A. P. Heuk at the University of Texas and University of Massachusetts-Amherst, respectively (24, 25). The cDNA for the pGEX-complexin 2 construct was a generous gift from T. Südhof at the Howard Hughes Medical Institute.

Reverse Transcription-PCR (RT-PCR)—Total RNA was isolated from brain and pancreas, and poly(A)-RNA was purified from pancreatic acinar cells. Tissues were homogenized in TRIzol using a Polytron. Total RNA was isolated using chloroform, isopropyl alcohol, and RNeasy mini kit from Qiagen. Acinar RNA was further purified using the Poly(A) Purist kit from Ambion. Primer pairs used were: complexin 1, 5′-ATGGAGTTCGTGATGAAACAAG-3′ (sense) and 5′-TTACTTCTTGAACATGTCCTGCA-3′ (antisense); and complexin 2, 5′-ATGGACTTCGTCATGAAGCA-3′ (sense) and 5′-TTACTTCTTGAACATGTCCTGCA-3′ (antisense). Reverse transcription of RNA was performed using the RETROscript kit from Ambion. PCR was performed on a MJ Mini Personal Thermo Cycler (Bio-Rad) with an initial denaturation of 94 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 51.3 °C for 1 min, 72 °C for 1 min amplification, and a final extension of 72 °C for 5 min.

Isolation of Pancreatic Lobules and Dispersed Acini—The University of Wisconsin Committee on Use and Care of Animals approved all studies involving animals. Pancreatic lobules were prepared by microdissection of an adult male Sprague-Dawley rat pancreas in HEPES buffer consisting of 10 mM HEPES, 137 mM NaCl, 4.7 mM KCl, 0.56 mM $MgCl_2$, 1.28 mM $CaCl_2$, 0.6 mM Na_2HPO_4, 5.5 mM D-glucose, 2 mM L-glutamine, and an essential amino acid solution. The buffer was supplemented with 0.1 mg/ml soybean trypsin inhibitor and 1 mg/ml BSA, gassed with 100% O_2, and adjusted to pH 7.48. Pancreatic acinar cells were isolated from adult male Sprague-Dawley rats by collagenase digestion as described previously (26). Acini were suspended in HEPES buffer and were maintained at 37 °C for 30 min before performing the assays.

Immunofluorescence Microscopy—After the indicated treatments, lobules or isolated acini were gently pelleted and fixed in 4% formaldehyde in 1× PBS for 2 h or 30 min, respectively, at room temperature. Lobules and acini were then dehydrated using a progressive sucrose gradient (27), resuspended in TissueTek O.C.T. compound, and then quick frozen in liquid nitrogen-cooled isopentane for cryosectioning. Immunofluorescence microscopy was conducted on 9-μm-thick cryostat sections as detailed previously (27, 28). The buffer used for blocking and incubation steps contained 1× PBS, 3% bovine serum albumin, 2% goat serum, 0.7% cold-water fish skin gelatin, and 0.2% Triton X-100. Sections were incubated with anti-VAMP 8 polyclonal antibody (1:20), anti-VAMP 2 monoclonal antibody (1:20), or anti-complexin 1,2 polyclonal antibody (1:20). Rhodamine or Alexa Fluor 647-conjugated phalloidin was added at 1 unit/200 μl or 10 units/200 μl, respectively, in 1× PBS for 20 min at room temperature after secondary incubation and rinsing. For dual immunofluorescence measurements, fluorophores were individually excited at the appropriate wavelength to ensure no overlapping excitation between

channels. Brightfield images were captured using a Nikon Eclipse TE2000 microscope, a PlanApo ×100 oil objective with a numerical aperture of 1.4, and a Hamamatsu Orca camera. Images were deconvolved by using Volocity software and were processed with Volocity, ImageJ, or Photoshop software.

Quantification of Immunofluorescence Images—Multiple brightfield, *z*-series images from at least three separate tissue preparations were analyzed using ImageJ software with the colocalization threshold plug-in. Threshold values were determined automatically by the software and, therefore, were unbiased and provided conservative estimates.

Tissue Fractionation—Acini were suspended in a lysis buffer containing 50 mM Tris (pH 7.4), 5 mM EDTA, 10 mM tetrasodium pyrophosphate, 1 mM PMSF, 1.28 mM benzamadine, and protease inhibitor mixture and sonicated. Soluble and membrane fractions were separated by centrifugation at 100,000 × *g* for 30 min at 4 °C. The pellet was resuspended in the same buffer containing 0.2% Triton X-100 and sonicated. ZG membranes were purified by Percoll density centrifugation as described previously (22).

Acinar Cell Permeabilization—Acini were suspended in a permeabilization buffer containing 20 mM PIPES (pH 6.6), 139 mM potassium glutamate, 4 mM EGTA, 1.78 mM $MgCl_2$, 2 mM MgATP, 0.1 mg/ml soybean trypsin inhibitor, 1 mg/ml bovine serum albumin, and 35 pM PFO. PFO is a cholesterol-dependent cytolysin that assembles to create large (25-nm) aqueous pores in cell membranes (24, 25). PFO was allowed to bind to intact cells on ice for 15 min, and excess unbound PFO was removed by washing at 4 °C in the same buffer without PFO. Acini were aliquoted into prechilled microcentrifuge tubes containing the indicated amounts of recombinant proteins. The cell suspension was then diluted with an equal volume of the same buffer. Cell suspensions were immersed in a 37 °C water bath and incubated with gentle mixing for 15 min. To the cell suspension, the indicated amounts of recombinant protein and the same buffer containing enough $CaCl_2$ to create the desired final concentration of free Ca^{2+} were added. The quantity of $CaCl_2$ added to the buffer was calculated on the basis of dissociation constants using WEBMAXCLITE version 1.15 software. Cell suspensions were immersed in a 37 °C water bath and incubated with gentle mixing for 30 min. Cells were then cooled in an ice bath for 3 min and centrifuged at 12,000 × *g* for 1 min. Amylase activity in the medium was determined using a Phadebas assay kit. Data were calculated as the percent of total cellular amylase present in an equal amount of cells measured at the start of the experiment.

Glutathione S-Transferase (GST) Fusion Proteins—GST fusion proteins of complexin 2 and mutants were purified by glutathione affinity chromatography and either left on the beads or released by thrombin cleavage as described previously (29). Single-point mutations in complexin 2 were made using QuikChange Lightning Site-directed Mutagenesis Kit (Stratagene). The following primer pairs were used 5′-CAGGAGGAAGAGCTCAAGGCCAAACATGCCCGCATG-3′ (sense) and 5′-CATGCGGGCATGTTTGGCCTTGAGCTCTTCCTCCTG-3′ (antisense), and 5′-CATGGAAGCGGAACATGAGAAGTCCGGCAG-3′ (sense) and 5′-CTGCCGGACCTTCTCATGTTCCGCTTCCGCTTCCATG-3′ (antisense) for R48L and R59H, respectively. The double mutant (R48L/R59H) was made by two consecutive single-point mutations. A complexin 2 truncation containing amino acids 41–134 was made by using the Fast Link DNA Ligation kit (Epicenter Biotechnologies) and the following primers: 5′-GCTCTAGAGCATGCTGAGGCAGCAGGAGGAA-3′ (sense) and 5′-CCCAAGCTTGGGTTACTTCTTGAACATGTCCTGCAG-3′ (antisense). The sense strand contains an XbaI site and a new start site whereas the antisense strand contains a HindIII site.

GST Pulldown Assays—Acini were lysed in a buffer containing 150 mM NaCl (pH 7.4), 10 mM HEPES, 1 mM EGTA, 2 mM $MgCl_2$, 0.2 mM PMSF, and protease inhibitor mixture and sonicated. Soluble and membrane fractions were separated by centrifugation at 100,000 × *g* for 30 min at 4 °C. The resulting pellet was resuspended and sonicated in the same buffer containing 1% Triton X-100 and then incubated on ice for 10 min. The solubilized pellet was centrifuged at 100,000 × *g* for 10 min at 4 °C to further separate the soluble and insoluble membrane fractions. For the GST pulldown assays, 3 mg of the Triton X-100-soluble membrane fraction was added to 50 μg of GST fusion protein and brought up to a volume of 1 ml with lysis buffer containing 1% Triton X-100. Following a 2-h incubation at 4 °C, the beads were washed three times in lysis buffer containing 1% Triton X-100 at 4 °C, and then bound proteins were eluted in SDS sample buffer.

RESULTS

Complexin 2 Is Present in Pancreatic Acinar Cells—Initial studies using RT-PCR indicated that neuronal specific complexin 1 was present in brain and pancreas but absent in isolated pancreatic acinar cells (Fig. 1*A*). In comparison, complexin 2 was also present in brain and pancreas but was additionally observed in isolated acinar cells. Immunoblotting with an anti-complexin antibody revealed 2 bands of ~17 and 18 kDa in brain lysates corresponding to complexin 1 and 2, respectively (Fig. 1*B*). Complexin 2 was detected in cytosolic fractions and at lower levels in membrane fractions. Western blotting with two different polyclonal anti-complexin antibodies revealed the same results (data not shown). Specificity of the complexin antiserum was demonstrated by preabsorbing with complexin antigen prior to immunoblotting and immunofluorescence (supplemental Fig. 1). Similar to SNARE proteins in acini, a 50-fold greater amount of lysate protein and prolonged exposure of the blots were necessary to detect complexin immunoreactivity compared with brain lysates where SNAREs make up greater than 1% of total protein. Tissue fractionation further revealed that complexin 2 was absent in purified zymogen granule membranes (ZGMs), which are enriched in VAMP 2 and 8.

Complexin 2 Inhibits Ca^{2+}-stimulated Exocytosis—To begin to identify a functional role for complexin 2 in Ca^{2+}-stimulated digestive enzyme secretion, recombinant complexin 2 was introduced into PFO-permeabilized acinar cells, and its effects on Ca^{2+}-stimulated amylase release were evaluated (Fig. 2). Permeabilized acini were preincubated with various concentrations of complexin 2 for 15 min and then stimulated with 3 μM free Ca^{2+} for 30 min. Complexin 2 inhibited Ca^{2+}-stimulated secretion in a concentration-dependent manner, with 36%

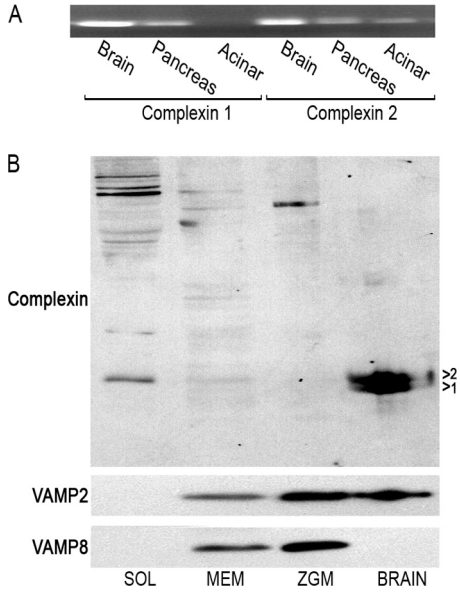

FIGURE 1. **Complexin 2 is expressed in acinar cells.** *A*, complexin 1 and 2 expression was analyzed by RT-PCR conducted on total RNA from whole pancreas and brain and poly(A)-RNA from isolated acini. Note that only complexin 2 is expressed in isolated acini. *B*, acinar-soluble (*SOL*) and membrane (*MEM*) fractions (100 μg/*lane*) were prepared from isolated acini, and ZGMs (50 μg) were prepared by Percoll density centrifugation. These fractions together with whole brain lysate (*BRAIN*) (2 μg) were analyzed by immunoblotting with anti-complexin (catalog number 122 102) and anti-VAMP antibodies (all at 1:1000). Note that complexin 2 is expressed in acini but not in ZGMs. Data are a single representative experiment performed three times on separate tissue preparations yielding identical results.

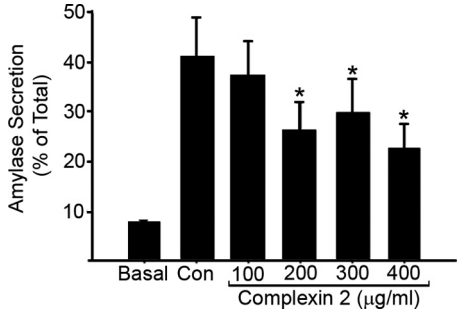

FIGURE 2. **Complexin 2 modulates Ca^{2+}-stimulated amylase release.** Isolated acini were permeabilized with PFO and preincubated with the indicated concentrations of recombinant complexin 2 for 15 min. Amylase secretion was measured after an additional 30 min under basal ($\leq$10 nM free Ca^{2+}) or Ca^{2+}-stimulated (3 μM) conditions. Secretion is expressed as a percentage of total cellular amylase measured at the start of the experiment. Basal secretion was not altered by maximal concentrations of complexin 2 protein. All data are the mean $\pm$ S.E. (*error bars*) of three independent experiments, each performed in triplicate. Statistical significance (*, $p < 0.03$) from control (*Con*) was determined using a paired *t* test.

inhibition achieved at 200 μg/ml and higher concentrations of up to 400 μg/ml further inhibiting secretion by 46% of control. This narrow concentration response to the complexin protein is similar to our previous studies in permeabilized acini using soluble SNARE proteins to inhibit secretion (22) or the proteins CRHSP-28 (26) and cysteine string protein (23) to augment secretion. Consistent with the ability of complexin 1 to inhibit exocytosis in other secretory cell types (18, 30), these findings suggest that high concentrations of complexin 2 in acini stabilize SNARE complex formation at a prefusion step, thereby inhibiting Ca^{2+}-stimulated secretion.

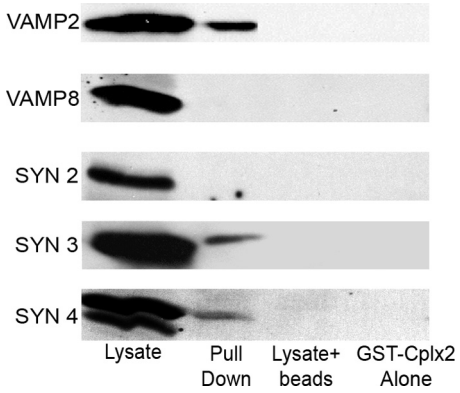

FIGURE 3. **Complexin 2 interacts with VAMP 2 and syntaxin 3 and 4 in acinar membrane fractions.** Total acinar membrane fractions were solubilized in buffer containing 1% Triton X-100, cleared to remove insoluble material, and incubated with GST-complexin 2 constructs immobilized on glutathione beads. Affinity-purified proteins were analyzed by immunoblotting with VAMP 2 or 8 and syntaxin 2, 3, or 4 antibodies (all at 1:1000). Note that GST-complexin 2 precipitated VAMP 2 and syntaxin 3 and 4 but did not interact with VAMP 8 or syntaxin 2. Data are a single representative experiment performed three times on separate tissue preparations yielding identical results.

Complexin 2 Interacts with VAMP 2, Syntaxin 3, and Syntaxin 4 in Pancreatic Acinar Cells—Pancreatic acini express VAMP 2 and 8 and syntaxin 3 located on ZGMs, and syntaxin 2 and 4 on the plasma membrane, whereas SNAP 23 is localized on both ZGMs and the plasma membrane (22, 31, 32). Hong *et al.* demonstrated that both VAMP 2 and 8 interact with syntaxin 4 and SNAP 23 to form a complete SNARE complex (31). Moreover, soluble forms of syntaxin 4 but not syntaxin 2 were able to inhibit Ca^{2+}-stimulated secretion from permeabilized cells, suggesting that syntaxin 4 on the plasma membrane is the primary isoform regulating secretion (22). Therefore, we used GST-complexin 2 pulldown assays to elucidate interactions between complexin 2 and acinar SNAREs. GST-complexin 2 bound to VAMP 2 but not VAMP 8 in acinar membrane fractions (Fig. 3). Additionally, GST-complexin 2 also interacted with syntaxin 3 and 4 but not syntaxin 2. Attempts to demonstrate a SNAP 23 interaction were precluded by high nonspecific interactions of the SNAP 23 antibody with recombinant complexin 2. Densitometric analysis indicated that GST-complexin 2 pulled down only 3.4 $\pm$ 0.4%, 2.0 $\pm$ 0.1%, and 1.0 $\pm$ 0.5% (mean $\pm$ S.E., $n = 3$) of the total VAMP 2, syntaxin 3, and syntaxin 4 present in the lysate, respectively. Attempts to coimmunoprecipitate complexin 2 with VAMP 2 or vice versa under numerous experimental conditions were unsuccessful, likely reflecting the small number of SNARE complexes formed under basal and stimulated conditions (100 pM CCK-8, 2 min) in acini. We did note that significant amounts of VAMP 2 were present in Triton X-100-insoluble fractions (1% v/v in lysis buffer), suggesting that some SNARE complexes may be lost when preclearing lysates prior to immunoprecipitation (data not shown). We previously reported similar difficulties in identifying acinar SNARE complexes by coimmunoprecipitations (22). These GST-complexin 2 pulldown assays suggest that complexin 2 interacts with SNARE complexes containing VAMP 2 and syntaxin 3 or syntaxin 4, whereas complexin 2 does not appear to interact with complexes containing VAMP 8 or syntaxin 2.

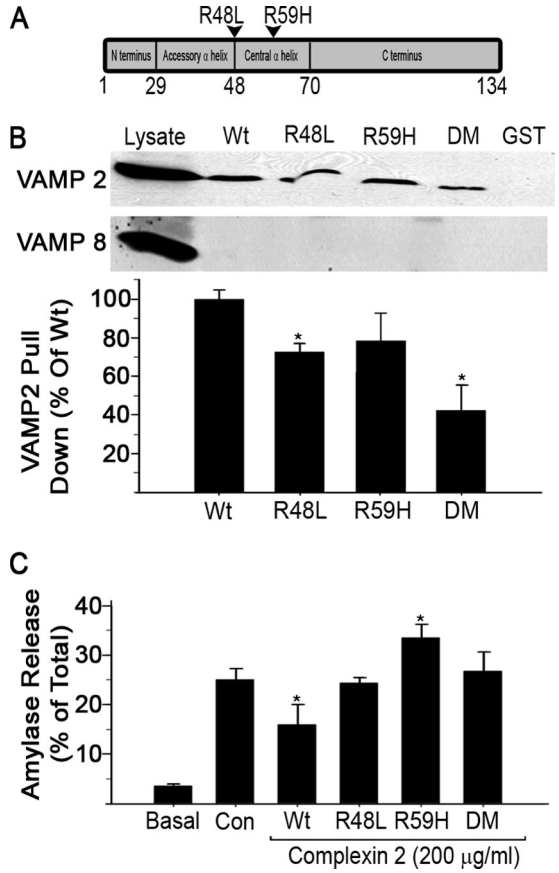

FIGURE 4. **Mutational analysis of complexin 2 SNARE binding and secretory function.** *A*, organization of complexin 2 functional domains is shown. Point mutations are indicated by *arrowheads* with amino acid changes listed. *B*, acinar membrane fractions were incubated with indicated GST-complexin 2 constructs and immunoblotted for VAMP 2 or 8 as detailed in Fig. 3. The amount of VAMP 2 pulled down was quantified by densitometric analysis of immunoblots from four independent experiments. *DM*, double mutant. Statistical significance (*, $p < 0.005$ from WT) was determined using a paired *t* test. *C*, permeabilized acini were incubated with recombinant complexin 2 constructs at 200 μg/ml as this concentration showed near maximal inhibition for WT complexin 2. Amylase secretion at 30 min was measured under basal ($\leq$10 nM free Ca^{2+}) or Ca^{2+}-stimulated (3 μM) conditions. Secretion is expressed as a percentage of total cellular amylase measured at the start of the experiment. All data are the mean $\pm$ S.E. (*error bars*) of three independent experiments each performed in triplicate. Statistical significance (*, $p < 0.05$) from control was determined using a paired *t* test.

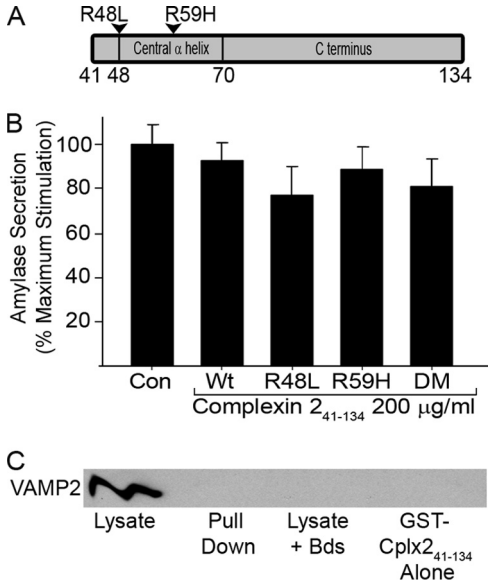

FIGURE 5. **Complexin 2_{41-134} truncation abolishes secretory function.** *A*, organization of complexin 2_{41-134} truncation is shown, with *arrowheads* indicating point mutations. *B*, permeabilized acini were preincubated with the indicated recombinant complexin 2 constructs at 200 μg/ml, and amylase secretion was measured under basal ($\leq$10 nM free Ca^{2+}) or Ca^{2+}-stimulated (3 μM) conditions. Secretion is expressed as a percentage of Ca^{2+}-stimulated amylase release measured in control cells. All data are the mean $\pm$ S.E. (*error bars*) of three independent experiments, each performed in triplicate. No statistically significant differences from control were detected for any of the constructs using paired *t* test. *C*, acinar membrane fractions were analyzed by GST-complexin 2_{41-134} pulldown assays and immunoblotted for VAMP 2. Note the loss of VAMP 2 binding of N-terminally truncated complexin 2.

Mutational Analysis of Complexin 2 SNARE Binding and Secretory Activity—The molecular interaction of complexin 2 with VAMP 2-containing SNARE complexes was further characterized by mutation of known complexin 1 SNARE interaction sites in neurons (17). Complexins are composed of an N terminus that is thought to promote membrane fusion, an accessory α-helix that is inhibitory to fusion, and a central α-helix that is necessary for SNARE binding (Fig. 4*A*). Xue *et al.* found reduced SNARE binding of complexin 1 when mutating arginine 48 to lysine (R48L) or arginine 58 to histidine (R59H) whereas the double mutant R48L/R59H inhibited SNARE binding completely (17). The R48L mutation is at the boundary of the accessory α-helix and central α-helix, whereas the R59H mutation is in the central α-helix. GST-complexin 2 pulldown assays revealed the R48L and R59H mutants modestly decreased VAMP 2 binding by 73 and 79%, respectively; however, the effects of the R59H mutant were not statistically significant (Fig. 4*B*). In contrast, the double mutant reduced VAMP 2 binding to 42% of that seen for wild type (WT). Analysis of the functional consequence of these mutations on secretion in permeabilized acini indicated that in contrast to WT, the R48L and double mutant lacked the ability to inhibit amylase secretion (Fig. 4*C*). Conversely, the R59H mutant consistently enhanced amylase secretion by 130% of control cells. This effect was seen in four separate acinar preparations and with two different batches of recombinant protein. Because the N terminus of complexin 1 was previously shown to be essential for enhancing SNARE-mediated membrane fusion *in vitro* (21), complexin 2 truncation constructs removing the N-terminal 40 amino acids (complexin 2_{41-134}) were also utilized (Fig. 5*A*). Unexpectedly, ablation of the N terminus abolished both the inhibitory activity of WT complexin 2 as well as the stimulatory activity of the R59H mutant (Fig. 5*B*). Consistent with this complete loss of secretory activity, GST-complexin 2_{41-134} was unable to interact with VAMP 2 in acinar membrane fractions (Fig. 5*C*).

Complexin 2 Colocalizes with VAMP 2 at the Apical Plasma Membrane—Immunofluorescence localization of complexin 2 was conducted on both pancreatic lobules and isolated acini to control for fixation artifacts sometimes seen in isolated acini. These artifacts occur due to the fragile nature of the acinar cells, making them prone to damage during fixation and labeling. Analysis of VAMPs in lobules confirmed previous studies, utilizing multiple VAMP 2 antibodies, that VAMP 2 containing granules accumulate in the most apical aspects of the cytoplasm immediately below the plasma membrane (22, 33, 34), whereas VAMP 8-positive granules are dispersed deeper within the api-

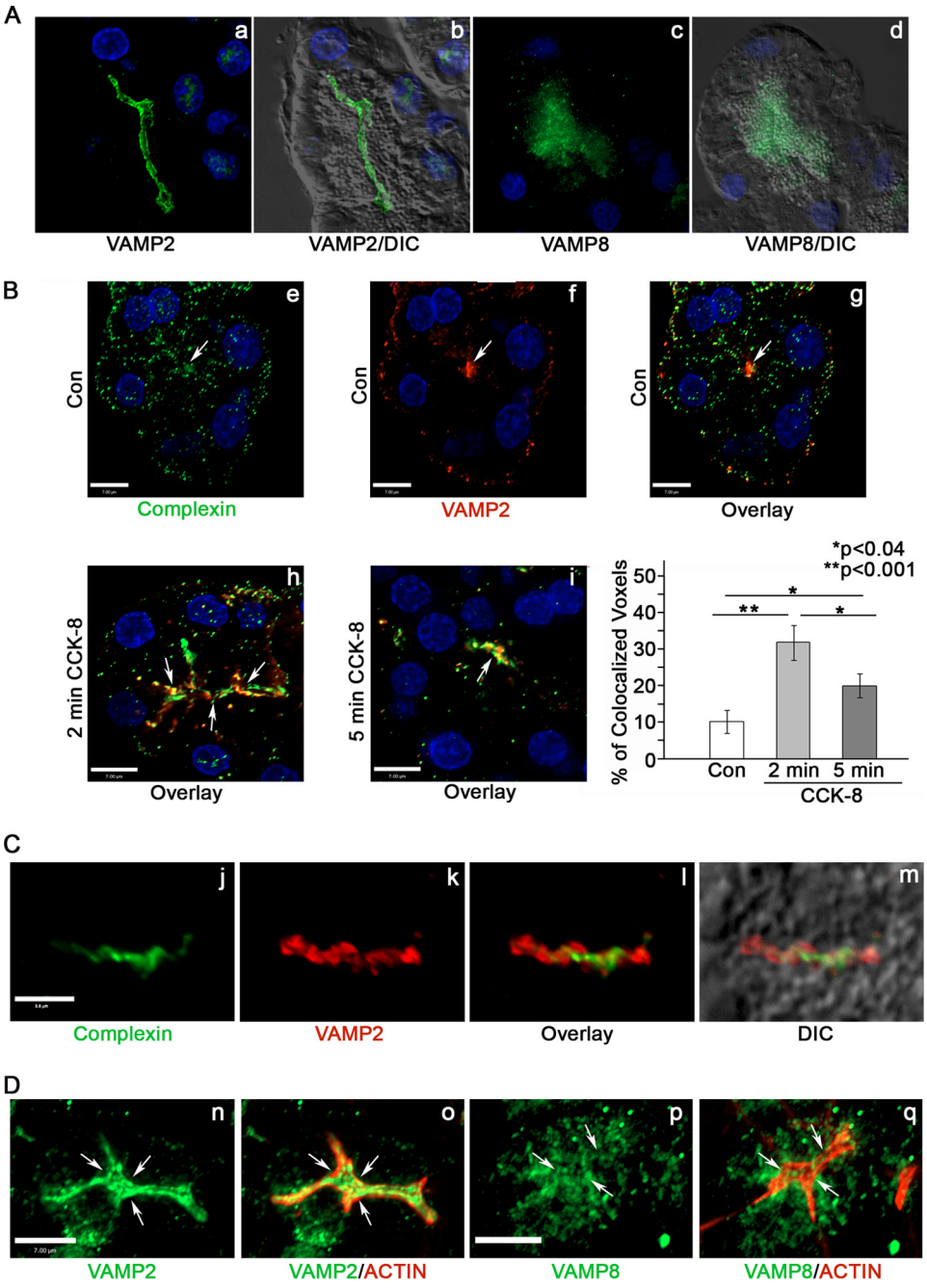

FIGURE 6. **Complexin 2 colocalizes with VAMP 2 at the apical plasma membrane.** Rat pancreatic lobules (*A*, *C*, and *D*) and rat pancreatic acini (*B*) were fixed in 4% paraformaldehyde. Brightfield immunofluorescence microscopy was conducted on 0.9-μm-thick cryostat sections (*A–D*). *A* and *D*, VAMP 2 and 8 antibody (1:50 and 1:20, respectively) immunoreactivity was detected using Alexa Fluor 488-conjugated anti-mouse IgG (1:250) and Alexa Fluor 488-conjugated anti-rabbit IgG (1:500), respectively. *A*, note the strong accumulation of VAMP 2 along the apical aspect of the acini. Also note the more expanded pattern of granule staining by VAMP 8. *B* and *C*, complexin (catalog number 122 002) and VAMP 2 antibodies (1:20 and 1:50, respectively) immunoreactivity was detected using Alexa Fluor 488-conjugated anti-rabbit IgG (1:500) and Alexa Fluor 546-conjugated anti-mouse IgG (1:500), respectively. *B*, acini were treated as control (*a–c*) and with 100 pM CCK-8 for 2 and 5 min (*d* and *e*, respectively). *Graph* in *B* shows quantitative analysis of complexin and VAMP 2 voxel colocalization acquired from multiple *z*-series images from three separate acinar cell preparations. Data are mean ± S.E. (*error bars*) ($n = 17$). *Scale bars*, 7 μm. Note the increased colocalization of complexin 2 with VAMP 2 after 2 and 5 min treatment with CCK-8. C, close-up colocalization of complexin and VAMP 2 at the acinar cell apex in control lobules is shown. *Scale bars*, 3.5 μm. *D*, in control pancreatic lobules, actin filaments (*ACTIN*) were identified with rhodamine-phalloidin, and VAMP 2 and 8 immunoreactivity was detected using Alexa Fluor 488-conjugated anti-mouse IgG (1:250) and Alexa Fluor 488-conjugated anti-rabbit IgG (1:500), respectively. *Scale bars*, 7 μm. Note the pronounced VAMP 2 staining above the actin filaments and along the apical membrane. In comparison, VAMP 8 staining is more expanded in the apical cytoplasm and very sparsely present at the apical membrane. *A* and *C*, images are a single representative experiment performed on two separate tissue preparations. Nuclei are labeled in *blue* with DAPI. Images show corresponding differential interference contrast results with a fluorescent overlay.

cal cytoplasm (Fig. 6*A*) (22). Analysis of complexin 2 in both isolated acini and lobules (Fig. 6, *B* and *C*, respectively) revealed a punctuate pattern of staining throughout the cytoplasm with a clear accumulation of immunoreactivity at the cell apex. Identical results were obtained with a second complexin 2 antibody (supplemental Fig. 1). Consistent with biochemical evidence of a complexin 2 interaction with VAMP 2, these molecules showed significant colocalization in apical regions of cells. When quantified by measuring colocalization of voxels obtained from multiple reconstructed *z*-series images, complexin 2 showed a modest 10% colocalization with VAMP 2 in control acini which increased significantly to 32% following 2 min CCK-8 (100 pM) stimulation (Fig. 6*B*). After 5 min of stimulation, colocalization declined to 20% but remained significantly higher than control.

Analysis of complexin 2 and VAMP 2 in control lobules confirmed their minimal overlap seen in isolated acini, indicating the molecules were juxtaposed at the cell apex (Fig. 6*C* and supplemental Movie 1). To identify the apical membrane more clearly, phalloidin staining of actin filaments located immediately below the plasma membrane was conducted, revealing significant overlap of VAMP 2 with the actin terminal web. Moreover, VAMP 2 was also extensively localized above the actin web along the plasma membrane, suggesting that these VAMP 2-positive granules were docked in a prefusion state (Fig. 6*D* and supplemental Movie 2). In comparison, VAMP 8 only modestly colocalized with actin filaments and was very sparsely dispersed above the actin terminal web along the apical plasma membrane (Fig. 6*D*).

Evaluation of complexin 2 and VAMP 2 at the apical membrane was achieved by triple labeling these molecules together with actin filaments in lobules (Fig. 7). High magnification of the cell apex again showed a 31.4% overlap of com-

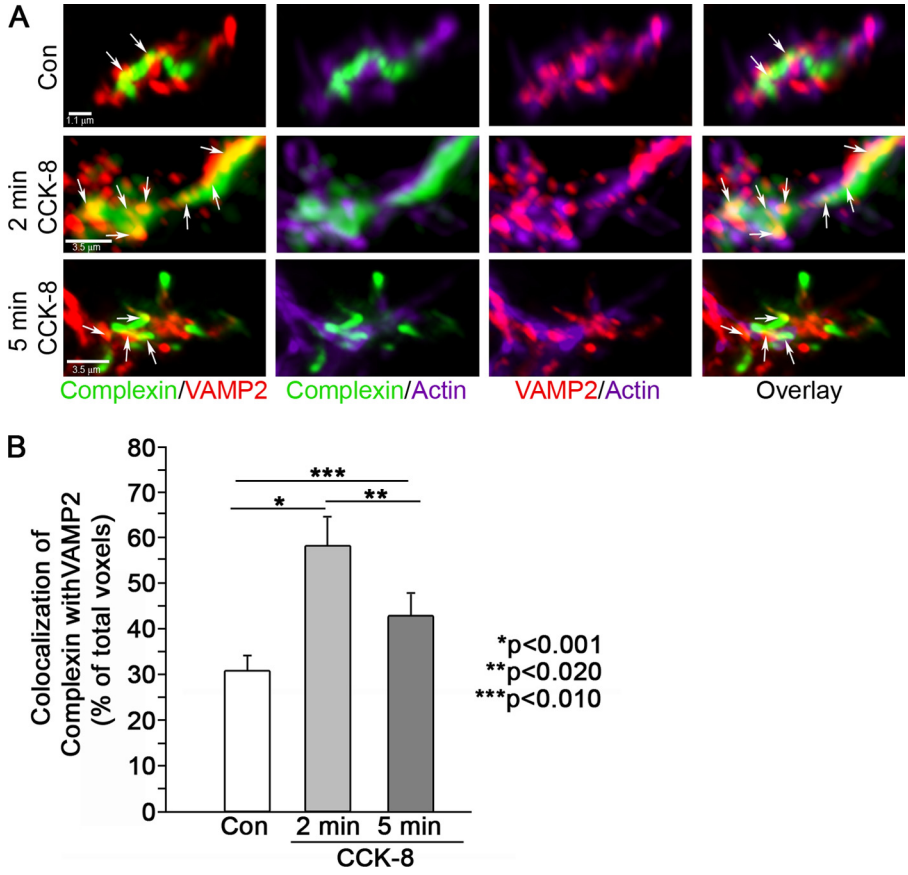

FIGURE 7. **Complexin/VAMP 2/actin colocalization at the apical aspect of pancreatic lobules.** *A*, rat pancreatic lobules were fixed in 4% paraformaldehyde. Brightfield immunofluorescence was conducted on 0.9-μm-thick cryostat sections. Complexin (catalog number 122 002) and VAMP 2 (1:20 and 1:50, respectively) immunoreactivity was detected using Alexa Fluor 488-conjugated anti-rabbit IgG (1:500) and Alexa Fluor 546-conjugated anti-mouse IgG (1:250), respectively. Actin localization was detected using phalloidin conjugated to Alexa 694 (5 units/200 μl). Pancreatic lobules were treated as control or at indicated times with 100 pM CCK-8. *Arrows* show areas of complexin and VAMP 2 colocalization. Note again the increase in colocalization between complexin and VAMP 2 after 2 and 5 min treatment with CCK-8. Scale bars in control (*Con*) indicate1.1 μm; in 2 min and 5 min CCK-8, they indicate 3.5 μm. Images are a single representative experiment performed on two separate tissue preparations. *B*, graph shows quantitative analysis of complexin and VAMP 2 voxel colocalization acquired from multiple *z*-series images from three separate acinar cell preparations. Data are mean ± S.E. (*error bars*) ($n = 16$).

plexin 2 and VAMP 2 at the plasma membrane under control conditions. VAMP 2 was typically present in ~1-μm punctate structures consistent with ZGs, whereas complexin 2 showed a more expanded localization along the plasma membrane. Upon 2 min stimulation with 100 pM CCK-8, the overlap of complexin 2 and VAMP 2 increased by 1.9-fold over control, although areas of complexin 2 staining independent of VAMP 2 were clearly evident (Fig. 7 and supplemental Movie 3). Areas of overlap between the molecules appeared in 1-μm spherical structures that were often present in a scalloped or stringed-bead arrangement, suggesting that multiple ZGs were fused at the plasma membrane. At 5 min after CCK-8 treatment, complexin 2 and VAMP 2 colocalization was diminished to 42%, similar to that seen in isolated acini. Interestingly, VAMP 2 staining at 5 min was less punctate and more expanded along plasma membrane regions. These data clearly support a functional interaction of complexin 2 with VAMP 2-containing SNARE complexes; however, the VAMP 2-independent localization of complexin 2 also suggests that it may interact with additional proteins, potentially t-SNARE complexes, present on the apical membrane.

DISCUSSION

The current findings establish a role for complexin 2 in acinar secretion and suggest that it interacts preferentially with ternary SNARE complexes containing VAMP 2, syntaxin 3, and syntaxin 4. Similar to the SNARE-clamping effects of complexin 1 in neurons (7, 35), complexin 2 inhibited Ca^{2+}-dependent secretion when introduced into permeabilized acini. Results that complexin 2 was most highly expressed in acinar cytosolic fractions with small amounts present in membranes are consistent with its immunofluorescence localization at the apical plasma membrane and absence on basolateral membranes. Moreover, the apical localization of complexin 2 together with SNARE proteins is clearly in line with a fundamental role for the protein in the secretory pathway of pancreatic acinar cells.

Demonstration that complexin 2 interacts with SNARE complexes containing VAMP 2 and syntaxin 4 is in agreement with previous studies showing that GST-VAMP 2 interacts with syntaxin 4 in acini (31, 32, 36). Additionally, we reported previously that syntaxin 4 is the primary syntaxin isoform regulating Ca^{2+}-dependent secretion, whereas syntaxin 2 is involved mainly in basal secretion (22). However, the precise role of syntaxin 4 in acinar secretion remains controversial, as Pickett *et al.* (37) reported that syntaxin 2 rather than syntaxin 4 is the major syntaxin for secretion. These results are partially supported by evidence that syntaxin 2 coimmunoprecipitated with VAMP 2 from acinar cell membranes (36). Moreover, when evaluated *in vitro* using recombinant proteins, no association of complexin 1 or 2 with SNARE complexes containing syntaxin 4 was found (13, 21), suggesting that the interaction with syntaxin 4 seen in acini may involve additional regulatory proteins.

The association of GST-complexin 2 with syntaxin 3 is intriguing because this SNARE primarily localizes to ZGs in acini (32) and has been implicated in ZG-ZG compound exocytosis (38). Further, syntaxin 3 was shown to coimmunoprecipitate VAMP 8 but not VAMP 2 in acini (36). Complexin 2 did not copurify with ZGs, indicating that its association with syntaxin 3 likely occurs transiently during secretory stimulation. These findings together with evidence that complexin 2 local-

ization is clearly present along apical membrane regions independent of VAMP 2 support that complexin 2 also participates in either ternary SNARE complexes independent of VAMP 2 and/or binary complexes involving SNAP 23 and syntaxin 3 or 4 (see below). Because acini express multiple isoforms of syntaxins including 2, 3, 4, 7, and 8 within the secretory pathway (31), more detailed genetic deletion studies will be necessary to delineate fully the precise role of each molecule as well as their potential regulation by complexin 2.

Acinar cells express two major isoforms of vesicle SNAREs, VAMP 2 and 8, which localize to ZGs (22, 31). Our findings that GST-complexin 2 preferentially interacts with SNARE complexes containing VAMP 2 were clearly supported by their colocalization at the apical membrane under basal and stimulated conditions. Acinar secretion proceeds as an initial peak phase within the first minute of stimulation and then declines to a plateau phase over several minutes, which is sustained in the presence of secretagogue (39). This pattern of exocytosis was first described using sequential secretory measures in isolated cells (40) and later quantified at the cellular level by membrane capacitance measures and differential interference contrast microscopy (41). Localization of VAMP 2 at the most apical aspects of acini is consistent with our previous report that VAMP 2-positive ZGs mediate the initial secretory response (22). Results that complexin 2 also localizes to the apical membrane under basal conditions are compatible with the known SNARE-clamping activity of complexins in neurons which allows for the synchronous release of neurotransmitters (7, 35). Although evidence to support synchronous exocytosis in acini is lacking, it is conceivable that complexin 2 functions analogously outside the nervous system to support the initial rapid peak phase of secretion.

Consistent with Xue *et al.*, who characterized complexin 1 function in complexin 1/2 double knock-out neurons (17), the single mutant R48L and double mutant R48L/R59H of complexin 2 showed reduced SNARE binding and when introduced into permeabilized acini, lost the ability to inhibit Ca^{2+}-stimulated secretion. However, unlike complexin 1 in neurons where the single R59H mutation also reduced SNARE binding and secretory activity, this same mutation in complexin 2 showed only a small statistically nonsignificant reduction in SNARE binding yet significantly enhanced Ca^{2+}-stimulated secretion by 130% of control. Although these results are clearly in line with complexins having both inhibitory and stimulatory roles in exocytosis, they were nonetheless surprising as the central α-helical domain (amino acids 48–70) is thought to be necessary for SNARE binding but not sufficient to modulate secretion (17, 18). Because the N-terminal domain (amino acids 1–29) is thought to be essential to stimulate secretion, we removed this domain and the first 12 residues of the inhibitory domain of complexin 2 (complexin 2_{41-134}), which in neurons retains normal SNARE binding but fails to modulate secretion (17, 18). However, in acini the complexin 2_{41-134} truncation lost all SNARE binding and correspondingly all secretory activity.

Explanations for these differences in complexin SNARE binding and secretory activity in acini *versus* neurons are uncertain. Unlike studies conducted in complexin 1/2-deficient neurons (17, 18), the current experiments were performed in cells that express endogenous complexin 2. Thus, complexin 2 constructs added to lysates or permeabilized cells must compete with endogenous protein for SNARE binding. Although complexin 2_{41-134} contains the minimal SNARE binding domain, its affinity for the SNARE complex may be diminished compared with the endogenous WT protein.

The stimulatory effect of the R59H mutant remains enigmatic as this mutation is within the SNARE binding region and outside the stimulatory domain. Although compelling evidence demonstrates a stimulatory role for the N-terminal domain in neurons (17, 18), Malsam *et al.* reported that the C-terminal half of complexin 1 was necessary to stimulate liposome fusion *in vitro* (21). This suggests that complexins may have more structurally diverse effects on membrane fusion depending on the experimental approach. For example, it is important to consider that the stimulatory effects of complexin 1 in neurons are based largely on the concerted interactions of both complexin 1 and synaptotagmin 1 with the ternary SNARE complex during the final stages of membrane fusion (18). Liposome fusion studies by Malsam *et al.* (21) did not include synaptotagmin in the fusion reactions. Notwithstanding the obvious differences in SNARE isoform expression between neural and exocrine tissues, the calcium sensor(s) that drives exocytosis in acini as well as the accessory proteins that promote vesicle docking and priming are largely unknown. These uncertainties create considerable ambiguity when comparing acini with other secretory systems.

Our results that VAMP 2 accumulates along the plasma membrane and within the actin terminal web under basal conditions suggest that significant numbers of VAMP 2-positive ZGs are both docked at the plasma membrane and tethered to the subapical actin filaments. Indeed, Braun *et al.* previously demonstrated that disruption of actin filaments abolished the apical accumulation of VAMP 2-positive ZGs in acini (33). Complexin 2 was localized primarily inside the actin filaments and along the plasma membrane and showed clear colocalization with VAMP 2 particularly with CCK-8 stimulation. However, significant amounts of complexin 2 were also juxtaposed to VAMP 2 in apical regions. These results support that complexin 2 not only colocalized in ternary SNARE complexes containing VAMP 2, but also occupied adjacent membrane regions. Although we saw no interaction with VAMP 8 in biochemical assays and detected very sparse localization of VAMP 8 on the plasma membrane under basal conditions, we cannot fully rule out a complexin 2 association with an additional ternary SNARE complex. Evidence that complexins also bind with lower affinity to t-SNARE complexes composed of SNAP 25 and syntaxin *in vitro* (4, 8, 10) creates the additional possibility that these intermediates exist in intact cells. Thus, it is possible that complexin 2 associates with SNAP 23 and syntaxin 3 or 4 on the plasma membrane prior to ternary SNARE formation. The lack of availability of affinity-purified complexin 2 polyclonal antibodies for fluorophore conjugation precluded our ability to study its colocalization with polyclonal antibodies to VAMP 8, SNAP 23, and syntaxin 2, 3, or 4.

ZG exocytosis is a highly regulated process integral to the robust secretory capacity of the pancreas. Secretion is abso-

lutely dependent on elevated Ca^{2+} but is also modulated by a variety of additional cellular messengers (42). In addition to its essential role in secretion, aberrant alterations in Ca^{2+} signaling have been shown to precipitate the onset of experimental pancreatitis in rodents (43). These alterations include a complete inhibition of apical secretion, redirected ZG exocytosis at the basolateral membrane, and the mixing of proteolytic zymogens with lysosomal enzymes (44). The current study uncovers a unique regulatory mechanism governing Ca^{2+}-dependent secretion in acini and paves the way for future studies focused on better understanding the specific role of SNARE-mediated membrane fusion events in normal and disease states.

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