COMPLEXIN 2 MODULATES VESICLE ASSOCIATED MEMBRANE PROTEIN (VAMP) 2 REGULATED ZYMOCEN GRANULE EXOCYTOSIS IN PANCREATIC ACINI*

Michelle A. Falkowski, Diana D.H. Thomas, and Guy E. Groblewski
From the Department of Nutritional Sciences
University of Wisconsin, Madison, Wisconsin 53706
Running head: Complexin 2 and acinar secretion
Address correspondence to: Guy E. Groblewski, Ph.D., Dept. of Nutritional Sciences, University of Wisconsin, 1415 Linden Drive, Madison, WI 53706. Tel.: 608-262-0884; Fax: 608-262-5860; E-mail: groby@nutricia.wisc.edu

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 pre-fusion state and also 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 t-SNARE complexes. Far less is understood about complexin’s 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 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 alpha helical domain reduced complexin 2 SNARE binding and concurrently abolished its inhibitory activity. Surprisingly, mutation of arginine 59 to histadine within the central alpha 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 CCK-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. Complexin 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 sequence 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 4 helical bundle composed of vesicle associated membrane protein (VAMP) 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 pre-fusion 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).

http://www.jbc.org/cgi/doi/10.1074/jbc.M110.146597 The latest version is at http://www.jbc.org. Published on September 9, 2010 as Manuscript M110.146597

Copyright 2010 by The American Society for Biochemistry and Molecular Biology, Inc.
Complexins are comprised of an N-terminus (aa 1-29), accessory alpha-helix (aa 30-48), central alpha-helix (aa 49-70) and a C-terminus (aa 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 alpha 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 alpha helix is essential for SNARE binding, the adjacent accessory alpha-helix has a negative effect and the N-terminus a positive effect on exocytosis (17). Maximov et al. more recently reported 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).

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 this isform 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 syntaxin 3 and 4. Use of site-specific and truncation mutants of complexin 2 regulatory domains in permeabilized cells indicate 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 (cat # 122 002 and 122 102), monoclonal anti-VAMP 2 (cat # 104 211), and polyclonal anti-syntaxin 2, 3 and 4 (cat # 110 123, 110 033, 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 (cat # ab79180) was purchased from Abcam.

**Other Reagents.** Soybean trypsin inhibitor, benzamidine, phenylmethanesulfonyl fluoride, 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 cocktail containing AEBSF, aprotinin, EDTA, leupeptin, and E64 from Calbiochem. Phadebas amylase assay kit, chloroform and isopropanol were purchased from Fisher Scientific. Alexa 488 and 546 conjugated rabbit and mouse secondary antibodies, Rhodamine and Alexa 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 (DSS) 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 pGEX-Complexin 2 construct was a generous gift from T. Südhof at the Howard Hughes Medical Institute.

Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from brain and pancreas and poly(A)-RNA was purified from pancreatic acinar cells. Tissue was homogenized in Trizol using a Polytron. Total RNA was isolated using chloroform, isopropanol and RNAeasy mini kit from QIAGEN. Acinar RNA was further purified using the Poly(A) Purist kit from Ambion. Primer pairs used were complexin 1 5’-ATGGAGTTCTGTGAACAACAG-3’ (sense) and 5’-TTACTTTCTGTATCCTCGCA-3’ (antisense) and complexin 2 5’-ATGGACTTCGTCATGAAGCA-3’ (sense) and 5’-TTACTTTCTGTATCCTCGCA-3’ (antisense). Reverse transcription (RT) of RNA was performed using the RETROscript kit from Ambion. Polymerase chain reaction (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 sec, 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 (in mM) 10 HEPES, 137 NaCl, 4.7 KCl, 0.56 MgCl₂, 1.28 CaCl₂, 0.6 Na₂HPO₄, 5.5 D-glucose, 2 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% O₂, 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 assays.

Immunofluorescence Microscopy. After indicated treatments, lobules or isolated acini were gently pelleted and fixed in 4% formaldehyde in 1x 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: 1x 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 647 conjugated phalloidin were added at 1U/200 µL or 10 U/200 µL, respectively, in 1x 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 X100 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, Image J, or Photoshop software.

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

Tissue Fractionation. Acini were suspended in a lysis buffer containing (in mM) 50 Tris (pH 7.4), 5 EDTA, 10 tetrasodium pyrophosphate, 1 PMSF, 1.28 benzamidine and protease inhibitor cocktail 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. Zymogen granule membranes were purified by Percoll density centrifugation as previously described (22).
Acinar Cell Permeabilization. Acini were suspended in a permeabilization buffer containing (in mM) 20 PIPES (pH 6.6), 139 K+-glutamate, 4 EGTA, 1.78 MgCl₂, 2 Mg-ATP, 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 CaCl₂ to create the desired final concentration of free Ca²⁺ were added. The quantity of CaCl₂ added to the buffer was calculated on the basis of dissociation constants using WEBMAXCLITE v1.15 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 previously described (29). Single point mutations in complexin 2 were made using QuikChange Lightning Site-Directed Mutagenesis Kit (STRATAGENE). The following primer pairs were used 5'-CAGGAGGAAGAGCTCAAGGCCAAACATGC CCGCATG-3' (sense) and 5'-CATGCGGGCATTTGCCCCTGTGCACGCTCTTC CTCCGTG-3' (antisense) and 5'-CATGGAAGCGGAACATGAGAAGTCCGGCAG G-3' (sense) and 5'-CTGCCGGACCTTCTCATGTTCCGCAGGTC TCCATG-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 (Epicentre Biotechnologies) and the following primers 5'-GCTTACAGCTGTCATGCGCCAGGAA-3' (sense) and 5'-CCCAAGCTTACTTCTGTAACATGTCCTGCAG-3' (antisense). The sense strand contains an XbaI site and a new start site while the antisense strand contains a HindIII site.

RESULTS

Complexin 2 is Present in Pancreatic Acinar Cells. Initial studies using reverse transcription-PCR (RT-PCR) indicated that neuronal specific complexin 1 was present in brain and pancreas but absent in isolated pancreatic acinar cells (Fig 1A). In comparison, complexin 2 was also present in brain and pancreas but was additionally observed in isolated acinar cells (Fig 1A). 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 approximately 17 and 18 kDa in brain lysates corresponding to complexin 1 and 2, respectively (Fig 1B). 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 was necessary to detect complexin immunoreactivity in comparison to brain lysates where SNAREs make up greater than 1% of total protein. Tissue fractionation further revealed complexin 2 was absent in purified zymogen granule membranes (ZGM) 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 evaluated. Permeabilized acini were preincubated with various concentrations of complexin 2 for 15 min and then stimulated with 3µM free Ca\(^{2+}\) for 30 min. Complexin 2 inhibited Ca\(^{2+}\)-stimulated secretion in a concentration-dependent manner with 36% 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.

**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 ZGM 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 syntaxin 4 on the plasma membrane is the primary isoform regulating secretion (22). Therefore, we used GST-complexin 2 pull-down 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 non-specific interactions of the SNAP 23 antibody with recombinant complexin 2. Densitometric analysis indicated that GST-complexin 2 pulled-down only 3.4 ± 0.4%, 2.0 ± 0.1% and 1.0 ± 0.5% (mean ± 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 TX-100 (1% vol/vol in lysis buffer) insoluble fractions 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 pull-down 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.

**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 alpha helix that is inhibitory to fusion, and a central alpha helix that is necessary for SNARE binding (Fig 4A). 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 (DM) inhibited SNARE binding completely (17). The R48L mutation is at the boundary of the accessory alpha helix and central
alpha helix, while the R59H mutation is in the central alpha helix. GST-complexin 2 pull-down 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. In contrast, the DM 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 DM lacked the ability to inhibit amylase secretion (Fig 4C). Conversely, the R59H mutant consistently enhanced amylase secretion by 130% of control cells. This effect was seen in 4 separate acinar preparations and with 2 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 5A). 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 5B). 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 5C).

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 in order 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 apical cytoplasm (Fig 6A) (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 6B). 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 6C, and Supplemental movie 2). To more clearly identify the apical membrane, 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 these VAMP 2 positive granules were docked in a prefusion state (Fig 6D, Supplemental movie 3). 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 6D).

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 complexin 2 and VAMP 2 at the plasma membrane under control conditions. VAMP 2 was typically present in approximately 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 4). Areas of overlap between the molecules appeared in 1 µm spherical structures that were often times present in a scalloped or stringed-bead arrangement suggesting multiple ZGs were fused at the plasma membrane. At 5 min post-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 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 preferentially interacts 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 are in agreement with previous studies showing that GST-VAMP 2 interacts with syntaxin 4 in acini (31,32,36). Additionally, we previously reported 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 as 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 co-purify with ZGs, indicating that its association with syntaxin 3 likely occurs transiently during secretory stimulation. These findings together with evidence that complexin 2 localization 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 fully delineate the precise role of each molecule as well as their potential regulation by complexin 2.

Acinar cells express 2 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 min of stimulation and then declines to a plateau phase over several min, 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 DIC 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 knockout 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 \( \text{Ca}^{2+} \)-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 \( \text{Ca}^{2+} \)-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 alpha 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\text{41-134}), which in neurons retains normal SNARE binding but fails to modulate secretion (17,18). However, in acini the complexin 2\text{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\text{41-134} contains the minimal SNARE binding domain, its affinity for the SNARE complex may be diminished compared to 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 exists demonstrating 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 \textit{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 to 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 sub-apical 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 primarily localized 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. Though 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 \textit{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.

Zymogen granule exocytosis is a highly regulated process integral to the robust secretory capacity of the pancreas. Secretion is absolutely 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.

REFERENCES


FOOTNOTES

*This work was supported by National Institutes of Health Grant DK07088 and a USDA HATCH grant WISO4958 to G.E. Groblewski.

The abbreviations used are: ZG, zymogen granule; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; VAMP, vesicle associated membrane protein, PFO, Perfringolysin O; GST, glutathione S-transferase.

FIGURE LEGENDS
Fig. 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 zymogen granule membranes (ZGM) (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 (cat # 122 102) and anti-VAMP antibodies (all at 1:1000). Note 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.

Fig. 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 (≤10 nM free Ca\(^{2+}\)) or Ca\(^{2+}\)-stimulated (3µM) conditions. Secretion is expressed as a % 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 and S.E. of three independent experiments each performed in triplicate. Statistical significance (*, p <0.03) from control was determined using a paired t-test.

Fig. 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% TX-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.

Fig. 4. Mutational analysis of complexin 2 SNARE binding and secretory function. A: Organization of complexin 2 functional domains. 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. Statistical significance (*, p <0.005) from wild type (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 (≤10 nM free Ca\(^{2+}\)) or Ca\(^{2+}\)-stimulated (3µM) conditions. Secretion is expressed as a % of total cellular amylase measured at the start of the experiment. All data are the mean and S.E. of three independent experiments each performed in triplicate. Statistical significance (*, p <0.05) from control was determined using a paired t-test.

Fig. 5. A complexin 2\(_{41-134}\) truncation abolishes secretory function. A: Organization of complexin 2\(_{41-134}\) truncation with arrowheads indicating point mutations. B: Permeabilized acini were preincubated with indicated recombinant complexin 2 constructs at 200 µg/ml and amylase secretion was measured under basal (≤10 nM free Ca\(^{2+}\)) or Ca\(^{2+}\)-stimulated (3µM) conditions. Secretion is expressed as a % of Ca\(^{2+}\)-stimulated amylase release measured in control cells. All data are the mean and S.E. 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\(_{41-134}\) pull-down assays and immunoblotted for VAMP 2. Note the loss of VAMP 2 binding of N-terminally truncated complexin 2.

Fig. 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 & D: VAMP 2 and 8 antibodies (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 & D). 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 & C: Complexin (cat # 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 (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 and S.E. (n = 17). Bars, 7 µm. Note the increased colocalization of complexin 2 with VAMP 2 after 2 and 5 min treatment with CCK-8. C: Shows a close-up colocalization of complexin and VAMP 2 at the acinar cell apex in control lobules. 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-rabbit IgG (1:250) and Alexa Fluor 488-conjugated anti-rabbit IgG (1:500), respectively. 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. Images (A & C) are a single representative experiment performed on 2 separate tissue preparations. Nuclei are labeled in blue with DAPI. Images (A & C) show corresponding differential interference contrast (DIC) images with a fluorescent overlay.

Fig. 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 (cat # 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. VAMP 2 and 8 localization was detected using phalloidin conjugated to Alexa 694 (5 U/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 min and 5 min treatment with CCK-8. Bars in control (1.1 µm), 2 min and 5 min CCK-8 (3.5 µm). Images are a single representative experiment performed on 2 separate tissue preparations. B: Graph showing quantitative analysis of complexin and VAMP 2 voxel colocalization acquired from multiple z-series images from three separate acinar cell preparations. Data are mean and S.E. (n = 16).
Fig 2

Amylase Secretion (% of Total)

Basal  Con  100  200  300  400
Complexin 2 (µg/ml)
Fig 4

A

<table>
<thead>
<tr>
<th>N terminus</th>
<th>Accessory α helix</th>
<th>Central α helix</th>
<th>C terminus</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>48</td>
<td>70</td>
</tr>
</tbody>
</table>

B

Lysate  Wt  R48L  R59H  DM  GST

VAMP 2

VAMP 8

VAMP2 Pull Down (% Of Wt)

<table>
<thead>
<tr>
<th></th>
<th>Wt</th>
<th>R48L</th>
<th>R59H</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAMP2 Pull Down (％ Of Wt)</td>
<td>100</td>
<td>80</td>
<td>70</td>
<td>60</td>
</tr>
</tbody>
</table>

C

Amylase Release (％ of Total)

<table>
<thead>
<tr>
<th>Basal</th>
<th>Con</th>
<th>Wt</th>
<th>R48L</th>
<th>R59H</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase Release (%)</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>30</td>
</tr>
</tbody>
</table>
Fig 5

A

B

Amylase Secretion (% Maximum Stimulation)

Con, Wt, R48L, R59H, DM

Complexin 2

200 μg/ml

C

VAMP2

Lysate, Pull Down, Lysate + Bds, GST-Cplx2

Alone
Fig 7

A

Con

2 min CCK-8

5 min CCK-8

Complexin/VAMP2 Complexin/Actin VAMP2/Actin Overlay

B

Colocalization of Complexin with VAMP2 (% of total voxels)

Con 2 min 5 min CCK-8

*p<0.001  **p<0.020  ***p<0.010
Complexin 2 modulates vesicle associated membrane protein (VAMP) 2 regulated zymogen granule exocytosis in pancreatic acini
Michelle A. Falkowski, Diana D. H. Thomas and Guy E. Groblewski

J. Biol. Chem. published online September 9, 2010

Access the most updated version of this article at doi: 10.1074/jbc.M110.146597

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2010/09/09/M110.146597.DC1