Identification of Secretory Granule Phosphatidylinositol 4,5-Bisphosphate-interacting Proteins Using an Affinity Pulldown Strategy*[S]

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Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) synthesis is required for calcium-dependent exocytosis in neurosecretory cells. We developed a PtdIns(4,5)P_2 bead pulldown strategy combined with subcellular fractionation to identify endogenous chromaffin granule proteins that interact with PtdIns(4,5)P_2. We identified two synaptotagmin isoforms, synaptotagmins 1 and 7; spectrin; α-adaptin; and synaptotagmin-like protein 4 (granuphilin) by mass spectrometry and Western blotting. The interaction between synaptotagmin 7 and PtdIns(4,5)P_2 and its functional relevance was investigated. The 45-kDa isoform of synaptotagmin 7 was found to be highly expressed in adrenal chromaffin cells compared with PC12 cells and to mainly localize to secretory granules by subcellular fractionation, immunoisolation, and immunocytochemistry. We demonstrated that synaptotagmin 7 binds PtdIns(4,5)P_2 via the C2B domain in the absence of calcium and via both the C2A and C2B domains in the presence of calcium. We mutated the polylysine stretch in synaptotagmin 7 C2B and demonstrated that this mutant domain lacks the calcium-independent PtdIns(4,5)P_2 binding. Synaptotagmin 7 C2B domain inhibited catecholamine release from digitonin-permeabilized chromaffin cells, and this inhibition was abrogated with the C2B polylysine mutant. These data indicate that synaptotagmin 7 C2B-effector interactions, which occur via the polylysine stretch, including calcium-independent PtdIns(4,5)P_2 binding, are important for chromaffin granule exocytosis. ***Molecular & Cellular Proteomics 6:1158–1169, 2007.***

Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2)[1] plays a critical role in neurosecretory cells for both the exocytosis and endocytosis of secretory vesicles (1–3). The PtdIns(4,5)P_2 required for secretion is synthesized on the plasma membrane (4), and the binding of vesicle-associated proteins to PtdIns(4,5)P_2 in *trans* is thought to be important for bringing the vesicle and plasma membranes together prior to exocytosis to ensure rapid and efficient fusion upon calcium influx (5). Putative PtdIns(4,5)P_2 effectors include synaptotagmin 1 (Syt1) (6, 7), calcium-activated protein for secretion (CAPS) (8, 9), and rabphilin (10).

CAPS binds to PtdIns(4,5)P_2 and is a potential PtdIns(4,5)P_2 effector for priming of secretory granule exocytosis (9). However, recent work on the CAPS1 knock-out mouse highlighted an involvement of CAPS1 in the refilling or storage of catecholamine in mature secretory granules therefore suggesting an alternative role for CAPS (11).

Syts play a fundamental role in triggering membrane fusion in a variety of systems, and much work has focused on Syt1 as the main candidate for calcium sensor in exocytosis (12–14). Syt1 binds to PtdIns(4,5)P_2, and this interaction is believed to play a critical role in bridging the secretory vesicle and plasma membranes during exocytosis (5, 6). However, multiple Syt isoforms are expressed in the central nervous system and neurosecretory cells, suggesting that different isoforms may be involved in modulating exocytosis through mechanisms such as homo- and hetero-oligomerization (7, 15, 16).

Among the Syts capable of modulating secretory granule exocytosis, Syt7 has generated controversy (13, 17). Syt7 was initially postulated to be a plasma membrane calcium sensor for exocytosis (18), although more recent studies have localized it to secretory granules in PC12 cells (17, 19). However, to date, Syt7 localization studies in PC12 cells have been carried out using overexpression studies. Furthermore RNA interference knockdown of endogenous Syt7 in PC12 cells has varying effects on secretion (17, 19) so the physiological significance of Syt7 in PC12 cells remains unclear. Likewise

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[1] The abbreviations used are: PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; CAPS, calcium-activated protein for secretion; CgA, chromogranin A; IPP, inositol polyphosphate; PtdIns, phosphatidylinositol; Syt, synaptotagmin; PtdIns(3,5)P_2, phosphatidylinositol 3,5-bisphosphate; VAMP, vesicle-associated membrane protein; ACTH, adrenocorticotropin; TBST, TBS with Tween 20; PH, pleckstrin homology; Syx/S25, syntaxin/SNAP-25.

the involvement of Syt7 in neurotransmitter release in the central nervous system remains to be determined. Syt7 knock-out mice do not have any obvious neurological defects (20); however, there is evidence that Syt7 splice variants can regulate the speed of synaptic vesicle recycling (21). Unlike Syt1, Syt7 is widely expressed, and there is compelling evidence that in non-neuronal cells Syt7 plays a role in lysosomal exocytosis, membrane resealing, and wound healing (20, 22–26).

In view of the critical role of PtdIns(4,5)P_2 in exocytosis, an important issue is to identify possible PtdIns(4,5)P_2 effectors involved in neurosecretion and to examine their role(s) in exocytosis. To address this we used a PtdIns(4,5)P_2 binding pulldown strategy combined with mass spectrometry to identify PtdIns(4,5)P_2-interacting proteins. Adrenal chromaffin cells are a widely used and well characterized system for studying regulated exocytosis. Highly purified secretory (chromaffin) granules from bovine adrenal chromaffin cells were therefore used as a source of protein to minimize the number of unrelated PtdIns(4,5)P_2-binding proteins. As expected, we positively identified Syt1 as a chromaffin granule PtdIns(4,5)P_2-interacting protein by mass spectrometry. Interestingly spectrin, α-adaptin, Syt7, and synaptotagmin-like protein 4 (granuphilin) were also identified, and these identifications were confirmed by Western blotting. Syt7 is endogenously expressed at high levels in chromaffin granules of chromaffin cells compared with PC12 cells, and using a mutational analysis, we demonstrated that the polylysine stretch in Syt7 C2B is involved in calcium-independent PtdIns(4,5)P_2 binding and is important for the inhibitory effect of Syt7 C2B on chromaffin granule exocytosis.

EXPERIMENTAL PROCEDURES

Subcellular Fractionation and Immunoisolation—Bovine adrenal medulla were isolated, homogenized, and subjected to differential centrifugation, and purified chromaffin granules were obtained as described previously (27, 28). Protein concentrations were determined by Bradford protein assay (Bio-Rad). PC12 cells were scraped into homogenization buffer (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 0.32 M sucrose) containing 1 mM DTT and Complete protease inhibitor mixture (Roche Applied Science) and homogenized by repeated passage through a 25-gauge needle, and cellular debris and nuclei were removed by low speed centrifugation. Membrane and cytosolic fractions were obtained by centrifugation at 125,000 $\times$ g_{av} for 45 min, and protein concentrations were determined as above. 50 μg of chromaffin and PC12 subcellular fractions were solubilized in Laemmli sample buffer and separated by SDS-PAGE, and proteins were detected by Western blotting with the indicated antibodies. For chromaffin granule immunoisolation experiments, freshly prepared, intact chromaffin granules (50 μg) were incubated for 2 h with polyclonal anti-Syt1 antibodies (a kind gift from P. Foran) against the cytoplasmic domain in the presence of 0.1% fatty acid-free BSA (Sigma). Protein A beads presaturated with 5% BSA were added for a further 2-h incubation. Beads were pelleted by gentle centrifugation and washed extensively. Associated proteins were analyzed by SDS-PAGE and Western blotting using anti-Syt7 antibodies (Synaptic Systems) and anti-chromogranin A antibodies (Neomarkers).

Phosphoinositide Pulldown—Chromaffin granules (250 μg) were solubilized in Buffer A (20 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 1 mM DTT) containing Complete protease inhibitor mixture (Roche Applied Science), phosphatase inhibitor mixtures I and II (Sigma), and 0.5% Nonidet P-40 at 2 mg/ml. Solubilized material was precleared by centrifugation for 15 min at 13,000 rpm at 4 °C and incubated for 3 h at 4 °C with either 1 nmol of biotinylated C_8-PtdIns(4,5)P_2 (Cell-Signals Inc., Columbus, OH) (29) prebound to UltraLink Plus NeutrAvidin beads (Pierce) or beads alone. Beads were washed extensively in Buffer A containing 0.5% Nonidet P-40 and then resuspended in Laemmli sample buffer containing β-mercaptoethanol. For calcium binding studies, chromaffin granules were solubilized in the presence of 4 mM EGTA or 4 mM EGTA with 100 μM free calcium, and beads were washed in the respective calcium buffers. Calcium concentrations were calculated using the WEBMAXC program (66). Proteins associated with the pulldowns were analyzed by Western blotting with antibodies raised against α/β spectrin (Chemicon), α-adaptin (BD Biosciences), granuphilin (30), synaptotagmin 1 (31), chromogranin A, synaptotagmin 7, and VAMP2 (Synaptic Systems). Recombinant proteins were also assayed for binding to the PtdIns(4,5)P_2 beads: 100 ng of eluted GST fusion protein was incubated with PtdIns(4,5)P_2 beads as above. Beads were washed thoroughly in the respective calcium buffers, and associated protein were analyzed by SDS-PAGE and Western blotting using anti-GST antibodies (Sigma).

In-gel Digestions—Gel slices were excised from gels and destained with two washes in 200 μl of 50% acetonitrile, 200 mM ammonium bicarbonate at 37 °C for 45 min. Destained gel pieces were dried in a centrifugal concentrator and reduced by rehydrating with 100 μl of 20 mM DTT, 25 mM ammonium bicarbonate followed by incubation at 65 °C under N_2. After discarding the supernatant the reduced gel slices were alkylated by incubating in 100 μl of 50 mM iodoacetamide, 25 mM ammonium bicarbonate at 37 °C for 40 min in the dark under N_2. Gel slices were washed with three changes of 200 μl of 25 mM ammonium bicarbonate at 37 °C for 15 min and redried. Trypsin digestion was performed by rehydrating dried gel slices in 20 μl of 50 ng/μl trypsin (Roche Applied Science modified sequencing grade) in 10% acetonitrile, 40 mM ammonium bicarbonate and incubating at room temperature for 1 h. An additional 50 μl of 10% acetonitrile, 40 mM ammonium bicarbonate was added, and the incubation continued for 18 h at 37 °C. Supernatant containing tryptic peptides was removed to a fresh tube, and peptides were extracted from the gel slice by three washes in 50 μl of 0.1% TFA at 37 °C for 45 min. Extracts were pooled and concentrated using ZipTips (Millipore) largely according to manufacturer's instructions with peptides eluted in 10 μl of 50% methanol, 0.1% TFA.

MALDI-TOF/TOF-MS/MS—Peptides were analyzed in a 4700 TOF/TOF Proteomics Analyzer (Applied Biosystems) operated in positive ion reflectron mode using a 355 nm neodymium-doped yttrium aluminium garnet (Nd:YAG) laser at 200 Hz. Samples were spotted 1:1 in 5 mg/ml α-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile, 0.1% TFA, 2.5 mM ammonium phosphate. MS data (2000 shots per sample spot) were calibrated using a plate wide external calibration using the 4700 Mass Standards kit (Applied Biosystems) containing des-Arg-bradykinin (MH^+ 904.458), angiotensin I (MH^+ 1296.685) Glu-fibrinopeptide B (MH^+ 1570.677), ACTH (1–17 clip, MH^+ 2093.087), ACTH (18–39 clip, MH^+ 2465.199), and ACTH (7–38 clip, MH^+ 3657.929). The 50 most intense peptides detected in each spot in MS mode using GPS Explorer (version 3.5, Applied Biosystems) were automatically selected for MS/MS analysis using 3000 laser shots at a laser power ~20% higher than that used for MS. MS/MS data were calibrated against the MS/MS fragments of the m/z = 1296.685 angiotensin I peptide in the standards.

Database Analysis—MALDI-TOF/TOF-MS/MS data were automatically analyzed using the GPS Explorer version 3.5 suite of software (Applied Biosystems). For each digest a combined MS and MS/MS analysis was performed using an in-house Mascot (version 1.9, Matrix

Science) search engine with the mammalian taxonomic subset of the Celera Discovery System database (KBMS2.1.2.20030813, 1,335,729 sequences, 427,926,375 residues) using 100-ppm MS peptide tolerance and 0.3-Da MS/MS tolerance. Searches allowed for fixed carbamidomethylated cysteine and variable methionine oxidation with no other post-translational modifications taken into account (32).

Immunocytochemistry—Bovine adrenal chromaffin cells prepared as described previously (28) were seeded on polylysine-coated coverslips. After 24 h cells were fixed in MeOH, rehydrated in PBS, and blocked in 3% BSA in PBS. Cells were incubated with primary antibodies in blocking buffer overnight at 4 °C, washed with PBS, incubated for 20 min at 22 °C with anti-rabbit Alexa488 and anti-mouse Alexa546 (Molecular Probes) in blocking buffer, washed, and mounted on slides using ProLong Gold (Molecular Probes). Samples were imaged by confocal microscopy (LSM 510, Zeiss). The degree of co-localization was calculated using Image pro+ software. Briefly puncta positive for Syt7 were outlined in the green channel, and the pixel density in both green and red channels was measured. Dual labeled puncta were considered to co-localize if the intensity in both the red and green channels was greater than 100 (scale 255).

Structural Modeling—The three-dimensional models of the wild-type and variant C2B domains of mouse synaptotagmin 7, residues 266–403 of the Q9R0N7 UniProt entry, and synaptotagmin 2, residues 273–420 of the P46097 UniProt entry, were built based on their homology to the C2B domain of rat synaptotagmin 1 (Protein Data Bank code 1k5w (33)) using SWISS-MODEL (34). Ramachandran plots as well as bonding, distribution, and packing of residues within the core were checked, and no major anomalies were found as expected from their high similarity to the template, around 49 and 89% sequence identity for Syt7 and Syt2, respectively.

Point Mutagenesis and Recombinant Protein Expression and Purification—Syt7 C2A and C2B clones in FLAG vectors were kindly provided by M. Fukuda (35) and recloned into PGEX-4T1. Point mutations of lysines 320, 321, and 325 were made using the QuikChange system (Stratagene) in PGEX-4T1. All clones were verified by DNA sequencing.

Recombinant proteins were expressed in BL21 bacteria under isopropyl 1-thio-β-D-galactopyranoside induction and bound to GSH-agarose beads (GE Healthcare). Beads were washed extensively with high salt buffer to remove impurities as described previously (36). GST fusion proteins were eluted with excess GSH, and protein concentrations were determined as above.

Phosphoinositide Dot-blot and Liposome Binding Assays—Lipids (100 nmol each) were spotted onto nitrocellulose membrane (Pall). Membranes were blocked in 5% fatty acid-free BSA in TBST and incubated with a 250 ng/ml concentration of the indicated GST fusion proteins overnight at 4 °C in blocking solution. Membranes were washed in TBST, and anti-GST antibodies (Sigma) were applied for 2 h at 22 °C in 1% BSA, TBST. After washing, membranes were incubated for 1 h at 22 °C with horseradish peroxidase-conjugated secondary antibodies (Pierce) and washed, and antibodies were detected with enhanced chemiluminescence (SuperSignal West Pico, Pierce). Large multilamellar vesicles containing synthetic dioleoylphosphatidylcholine, phosphatidylethanolamine, and PtdIns or PtdIns(4,5)P_2 (Avanti Polar Lipids) in a 70:25:5 molar ratio were prepared, and lipid binding experiments were carried out as described previously (36). Briefly 5 μg of recombinant protein was incubated with 40 μg of lipid for 10 min at 22 °C with agitation. Large multilamellar vesicles and associated proteins were collected by centrifugation and washed, and bound proteins were solubilized in Laemmli sample buffer and analyzed by SDS-PAGE and Coomassie Blue staining (n = 3 independent experiments). Bands were scanned and quantified by densitometry using Image J (National Institutes of Health, version 1.37; Supplemental Figs. 1 and 2). Significance was calculated using a one-tailed Student's t test. All lipids were stored at −20 °C under nitrogen in glass vials with Teflon closures. Multilamellar vesicles were prepared under nitrogen and used within 24 h.

Chromaffin Cell Release Experiments—Chromaffin cells were isolated, and release experiments on digitonin-permeabilized cells were performed as described previously using KGEP buffer: 139 mM potassium glutamate, 5 mM glucose, 5 mM EGTA, 20 mM PIPES-NaOH (pH 6.7) (28). Recombinant fusion proteins at the concentrations indicated were present in the permeabilization and stimulating buffers. Aliquots of the supernatant were taken, and catecholamine content was measured fluorometrically (37) using a Fluorostar Optima microplate reader. Catecholamine released was expressed as a percentage of total catecholamine content (n = 8; data shown are representative of three independent experiments).

RESULTS

Identification of Chromaffin Granule PtdIns(4,5)P_2-binding Proteins—PtdIns(4,5)P_2 is important for large dense core vesicle exocytosis in neurosecretory cells (4, 38, 39). Here we used a pulldown strategy using biotinylated PtdIns(4,5)P_2 bound to avidin-conjugated Sepharose beads to identify PtdIns(4,5)P_2-interacting proteins from chromaffin granules. PtdIns(4,5)P_2 plays a role in a multitude of cellular processes; therefore, to restrict the potential interacting proteins to those likely to be involved in exocytosis, we used solubilized proteins from purified chromaffin granules isolated from bovine adrenal medulla as our starting material. Following incubation of chromaffin granule proteins with PtdIns(4,5)P_2 beads, bound proteins were separated by SDS-PAGE and visualized by Coomassie Blue staining (Fig. 1*A*). Bands of interest were excised and subjected to in-gel tryptic digestion. Tryptic peptides were analyzed by mass spectrometry, and the results are shown in Table I and Supplemental Table I.

Protein scores obtained by searching databases with peptide mass data obtained from in-gel digests of bands shown in Fig. 1*A* indicated that the identities assigned to proteins in these bands (Table I) were not due to random matches of peptide masses. The MS/MS data obtained on peptide ions observed in the digests of bands 3 and 4 served to support the identities of proteins in these bands as granuphilin and synaptotagmin 1, respectively, obtained by peptide mass mapping.

Data obtained for gel band 1 suggests that α2 and β2 spectrin are present in this band. The presence of both β and α spectrin in band 1 reflects their similar molecular weights. Inspection of the peptide mass data indicates that, in general, the two proteins were identified using different peptide mass sets (Fig. 2, *A*, *B*, and *C*). Where peptide masses common to both proteins were observed, the sequences attributed to these masses were never the same (Supplemental Table I). The identification of spectrin is consistent with previous studies demonstrating the interaction of the spectrin PH domain with PtdIns(4,5)P_2 (40). Interestingly spectrin was also localized to chromaffin granules and implicated in exocytosis in an early study (41). Band 2, with apparent molecular mass of ~100 kDa, was identified as α-adaptin isoform A, consistent

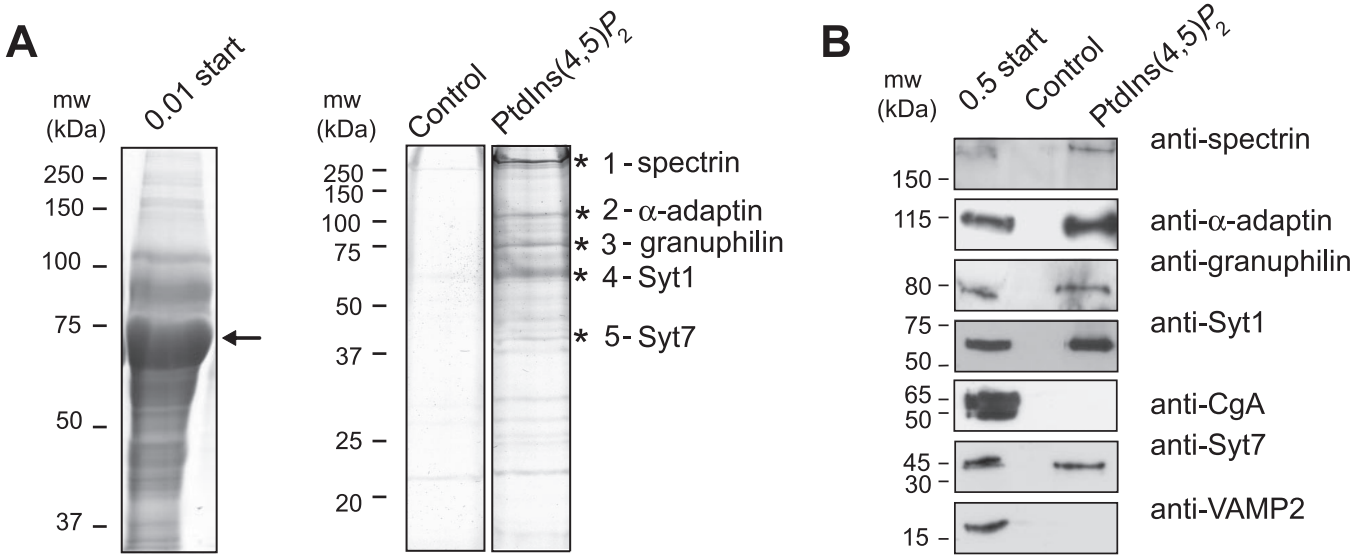


FIG. 1. **Identification of PtdIns(4,5)P_2-associated proteins.** *A*, chromaffin granule proteins associated with control or PtdIns(4,5)P_2 beads were separated by SDS-PAGE and visualized by Coomassie Blue staining. Bands specific to PtdIns(4,5)P_2 beads and positively identified by mass spectrometry are highlighted with *asterisks*. The *numbers* correspond to the protein identification data in Table I. 1% (*0.01 start*) of the start-ing material is shown for comparison, and the *arrow* indicates the position of chromogranin A. *B*, chromaffin granule proteins associated with control or PtdIns(4,5)P_2 beads and half of the start material (*0.5 start*) were separated by SDS-PAGE and Western blotting and probed using antibodies specific for the proteins indicated.

TABLE I

Proteins putatively identified by MALDI-TOF/TOF-MS of in-gel tryptic digests of the bands indicated in Fig. 1

Peptide mass (MS) and fragmentation (MS/MS) data were used to search against mammalian proteins in the Celera Discovery System database (KBMS2.1.2.20030813). Protein scores higher than 65 indicate with 95% confidence (protein score confidence interval (C.I.) that the proteins were not identified by random matches of peptide mass data.

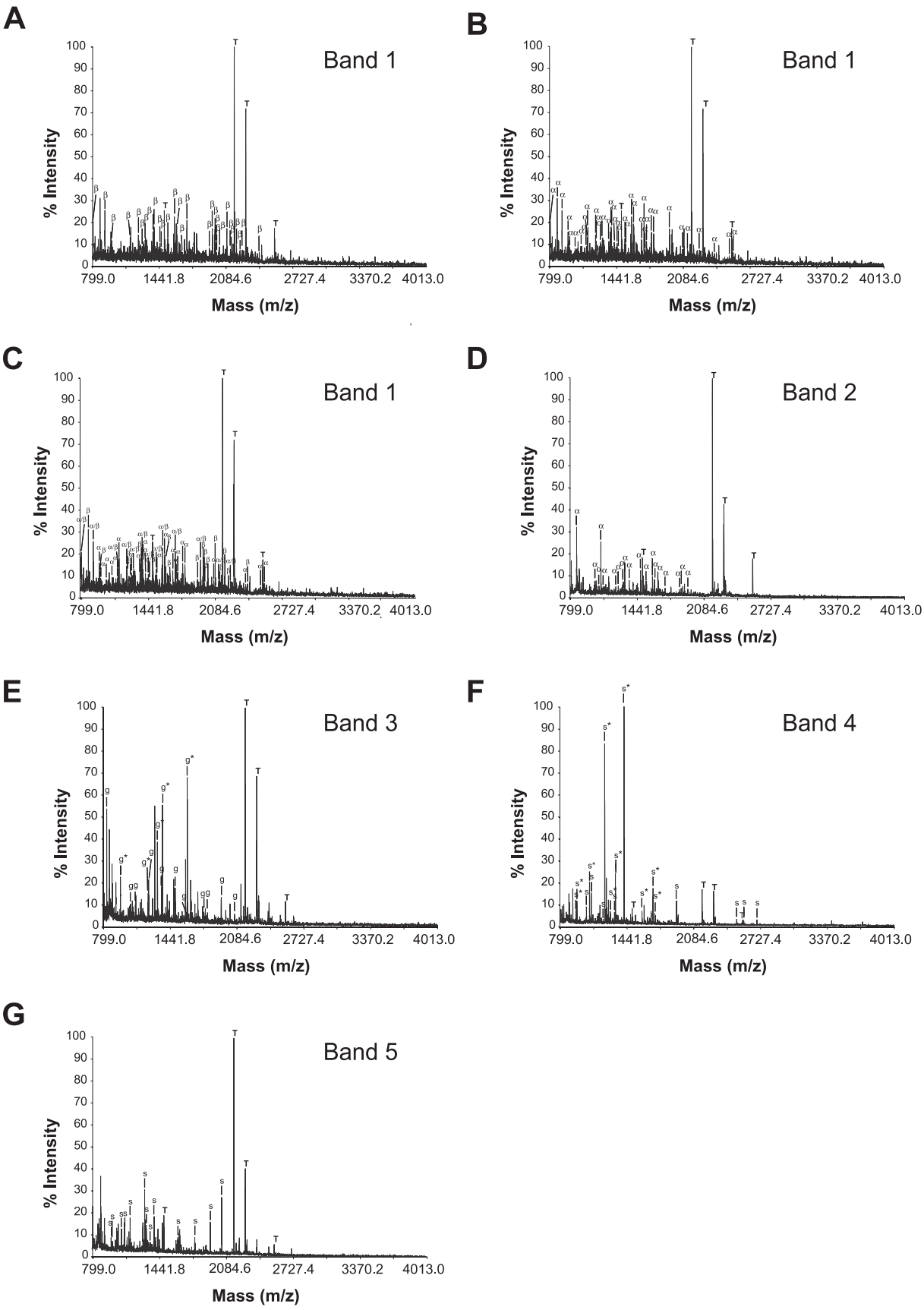
Gel band	Protein name	Species	Protein molecular weight	Protein score	Protein score C.I.	Peptide count[a]	Sequence coverage[b]	Accession no.
					%		%	
1	β2 spectrin	*Homo sapiens*	251,947.5	137	100	31	17.6	Trm|Q8IX99
1	α2 spectrin	*H. sapiens*	285,688.8	167	100	38	20.2	Gb|AAB41498.1
2	α-Adaptin A	*Mus musculus*	108,679.3	126	100	21	35.6	Spt|P17426
3	Synaptotagmin-like protein 4 (granuphilin)	*H. sapiens*	76,529	199	100	18 (3)	29.6	Spt|Q96C24
4	Synaptotagmin 1	*Bos taurus*	47,934.9	441	100	20 (3)	54.7	Spt|P48018
5	Synaptotagmin 7	*M. musculus*	45,785.8	124	100	13	39.2	Spt|Q9R0N7

[a] Peptide count for peptide in the spectra of tryptic digests (Fig. 2) that matched the proteins identified. Values in parentheses represent the number of peptides that also produced fragmentation data with ion scores of 30 or greater. Ion scores higher than 35 indicate with 95% confidence (total ion confidence interval) that peptide fragmentation data were not randomly matched to the identified protein. For proteins where no bovine sequence was available, human or mouse orthologues were returned as the most likely matches.

[b] Percentage of the protein amino acid sequence covered by all matching peptides.

with previous studies where α-adaptin was shown to interact with phosphoinositides including PtdIns(4,5)P_2 (42). α-Adaptin is part of the AP-2 clathrin adaptor complex required for clathrin-dependent endocytosis of synaptic vesicles (43) and has been localized to highly purified synaptic vesicles (44). Band 3 with approximate molecular mass of 75 kDa was identified as synaptotagmin-like protein 4 (granuphilin) with a high degree of confidence. Granuphilin has been localized to large dense core vesicles in PC12 cells (45) and contains two C2 domains that are potential phosphoinositide-interacting domains. Bands 4 and 5 were identified as synaptotagmin isoforms: band 4 was identified as Syt1, a known PtdIns-(4,5)P_2-binding protein (5, 6), and band 5 was found to be Syt7 (Table I).

The presence of the above mentioned identified proteins in the PtdIns(4,5)P_2 pulldowns was validated by Western blotting using specific antibodies (Fig. 1*B*). Pulldowns were also Western blotted with antibodies against chromogranin A and VAMP2, both abundant chromaffin granule proteins, and the lack of immunoreactivity associated with the PtdIns(4,5)P_2 beads highlighted the efficiency and selectivity of the pull-down strategy (Fig. 1*B*).



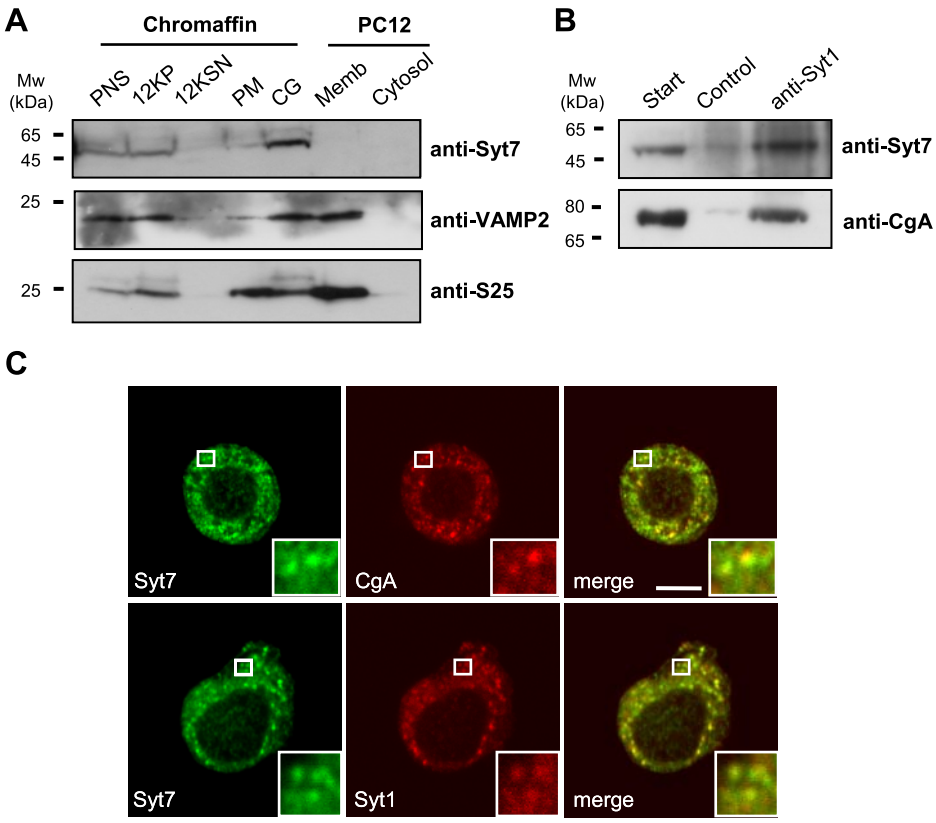


FIG. 3. **Synaptotagmin 7 localizes to chromaffin granules.** *A*, subcellular fractions of chromaffin cells and PC12 cells (50 μg) were analyzed by SDS-PAGE and Western blotting with antibodies against Syt7, the secretory vesicle protein VAMP2, and SNAP-25 (*S25*) as a predominantly plasma membrane protein. *PNS*, postnuclear supernatant; *12KP*, crude membrane fraction; *12KSN*, crude cytosolic fraction; *PM*, plasma-membrane enriched fraction; *CG*, chromaffin granule fraction; *Memb*, membrane. *B*, freshly isolated chromaffin granules (50 μg) were incubated with protein A beads alone (*Control*) or protein A beads with an antibody against the cytoplasmic domain of Syt1. Proteins associated with the beads were separated by SDS-PAGE and analyzed by Western blotting with anti-Syt7 and anti-CgA antibodies. Data shown are representative of three independent experiments. *C*, fixed chromaffin cells were used for co-localization experiments using anti-Syt7 antibodies (*green*) and antibodies against the chromaffin granule proteins Syt1 and CgA (*red*). Images are 0.5-μm confocal z-sections. *Scale bar*, 5 μm.

Chromaffin Granule Localization of Synaptotagmin 7—Of the PtdIns(4,5)P_2-binding proteins positively identified, the finding of Syt7 was interesting on two counts. First this is the first time binding of native, full-length Syt1 and Syt7 to PtdIns(4,5)P_2 has been demonstrated. Second there has been controversy in the literature as to whether Syt7 localizes to the plasma membrane or to secretory vesicles (17–19). To confirm that the Syt7 detected in the chromaffin granule preparation was not due to contaminating plasma membrane, fractions from the chromaffin cell subcellular fractionation and PC12 membrane and cytosolic fractions were separated by SDS-PAGE and Western blotted with anti-Syt7 antibodies (Fig. 3*A*). The anti-Syt7 antibody recognized a major 45-kDa band in chromaffin cells that was enriched in the chromaffin granule fraction (Fig. 3*A*). Our Western blot data and the 45-kDa band identified by mass spectrometry (Table I) are in good agreement with previous studies reporting that the 45-kDa splice variant is the predominant variant in mouse brain (46). Subcellular fractionation showed that Syt7 was detectable in the membrane-containing fraction (12KP), but only low levels were present in the crude cytosolic fraction (12KSN) comprising cytosol and light membrane fractions (including microsomes). Syt7 was barely detectable in the plasma membrane-containing fraction but was clearly enriched in the chromaffin granule fraction. Although we cannot exclude the presence of a pool of Syt7 on the plasma membrane, it is notable that Syt7 was not detectable in membrane or cytosolic fractions from our clone of PC12 cells (equivalent to the 12KP and 12KSN fractions for chromaffin cells; Fig. 3*A*). Western blotting using antibodies against SNAP-25, a predominantly plasma membrane protein, demonstrated that the 12KP and plasma membrane-containing fractions were enriched for SNAP-25. Some SNAP-25 was present in the chromaffin granule fraction consistent with previous reports (47), whereas VAMP2 was enriched in the chromaffin granule fraction (Fig. 3*A*).

To confirm that the presence of Syt7 in the chromaffin granule fraction was not due to contamination with other compartments, antibodies against the cytoplasmic domain of Syt1 were used to selectively immunoisolate intact, freshly prepared chromaffin granules (Fig. 3*B*). Western blotting using anti-Syt7 antibodies confirmed that chromaffin granules isolated with the anti-Syt1 antibody also contained Syt7. This

Fig. 2. **MALDI-TOF-MS spectra of identified proteins.** For each spectrum, ion peaks corresponding to peptides that matched the identified sequence are indicated. Peaks corresponding to trypsin autolysis products are indicated by *T*. Each panel is labeled with the gel band number indicated on Fig. 1*A*. *A*, *B*, and *C* correspond to data acquired from band 1 with β spectrin (β), α spectrin (α), and both β and α spectrin (α/β), respectively. *D*, *E*, *F*, and *G* correspond to data acquired from bands 2 (α-adaptin, α), 3 (granuphilin, *g*), 4 (synaptotagmin 1, *s*), and 5 (synaptotagmin 7, *s*). For bands 3 and 4, peptide ions for which subsequent MS/MS ion scores of greater than 30 were obtained are indicated as *.

demonstrates that different Syt isoforms are present on the same vesicles as shown previously for Syt1 and Syt2 on synaptic vesicles (15). Antibodies against chromogranin A (CgA), a soluble protein found inside chromaffin granules, were used as a positive control to verify that intact chromaffin granules were being isolated (Fig. 3*B*).

Finally the localization of Syt7 in chromaffin cells was confirmed by immunocytochemistry using anti-Syt7 together with anti-Syt1 or anti-CgA antibodies. Syt7 immunoreactivity localized to punctate structures that co-localized with both secretory granule markers, Syt1 and CgA (Fig. 3*C*). Syt7 immunostaining did not overlap completely with these markers consistent with the reported localization of Syt7 on lysosomes in fibroblasts (22) and in PC12 cells overexpressing Syt7 (17). Image analysis revealed that 94.6% of the Syt7-positive organelles co-localized with CgA (450 puncta monitored, seven cells), and 91.7% of the Syt7-positive organelles co-localized with Syt1 (150 puncta monitored, four cells). Altogether our data demonstrated that, in chromaffin cells, most endogenous Syt7 is present on secretory granules.

Calcium-dependent and -independent Synaptotagmin 7-PtdIns(4,5)P_2 Interactions—Previous data on Syt-PtdIns(4,5)P_2 binding have come from studies on the recombinant C2 domains. Although the interaction between Syt1 and PtdIns(4,5)P_2 was originally shown to be calcium-dependent (6), recent data suggest an important interplay between calcium-independent and calcium-dependent modes of PtdIns(4,5)P_2 binding (5). Syt7 C2A has been shown previously to bind PtdIns(4,5)P_2 in a calcium-dependent manner (7). Because the original PtdIns(4,5)P_2 pulldowns for mass spectrometry were performed in conditions where divalent cations were unbuffered, pulldowns were repeated in the absence (4 mM EGTA) or presence of calcium (4 mM EGTA, 100 μM free calcium) to determine whether endogenous Syt1 and Syt7 were interacting with the PtdIns(4,5)P_2 beads in a calcium-dependent manner. Western blotting demonstrated that both native Syt1 and Syt7 associate with PtdIns(4,5)P_2 beads in the absence and to a greater degree in the presence of calcium (Fig. 4*A*).

Three lysine residues from a polybasic region of the Syt2 C2B domain have been shown by mutagenesis to be critical for calcium-independent inositol polyphosphate (IPP) binding (48). Syt7 C2B also binds to IPP (49), and sequence comparisons of the C2B domains of Syt7 with Syt1 and Syt2 demonstrate that the three critical lysines are all present in the Syt7 C2B (Fig. 4*B*, *boxed* and *highlighted* in *red*). Modeling of the C2B domains of Syt7 and Syt2 shows that both domains present similar electrostatic characteristics on their surfaces (Fig. 4*C*). This includes the polybasic region suggested to be involved in calcium-independent binding to lipids despite the presence of a negatively charged residue at the beginning of strand 4 in Syt7. Likewise the three mutations that decrease IPP binding capability in Syt2 result in a similar change of properties in Syt7, namely an alteration of the electrostatic

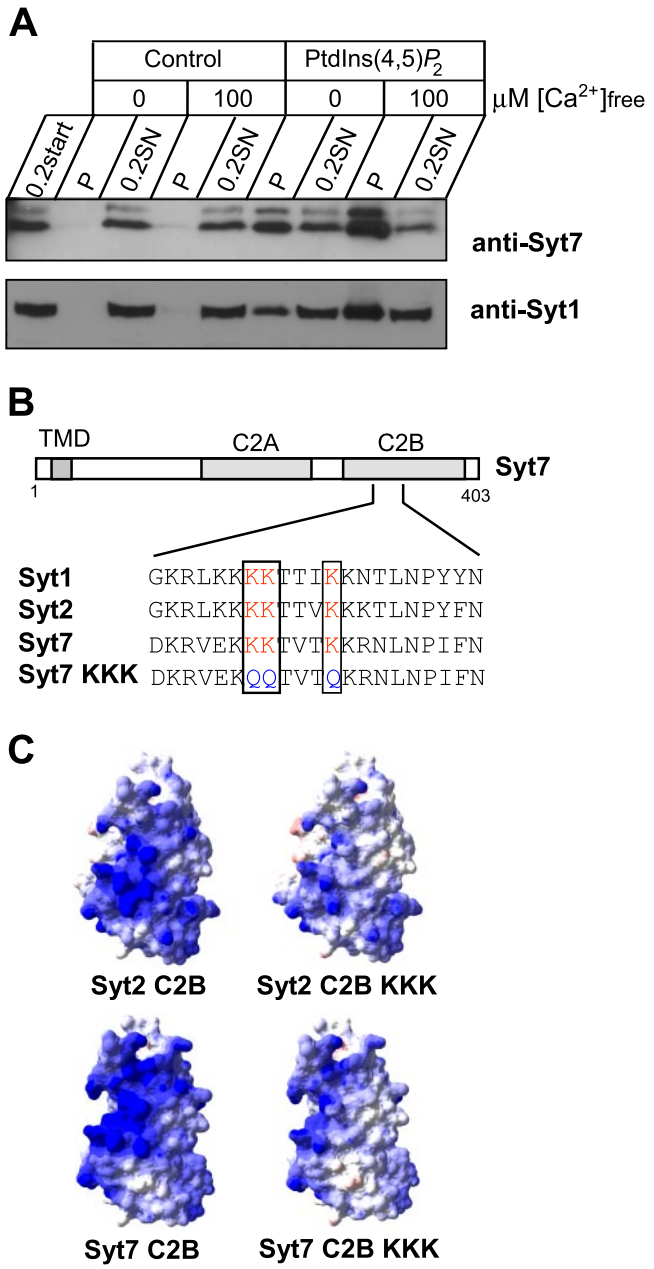


FIG. 4. **Synaptotagmin 7 binding to PtdIns(4,5)P_2 increases in a calcium-dependent manner.** *A*, control or PtdIns(4,5)P_2 beads were incubated with solubilized chromaffin granule proteins (250 μg) in the presence or absence of 100 μM free Ca^{2+}. Proteins associated with the beads (*P*) and one-fifth of the starting material (*0.2start*) and the supernatant after pulldown (*0.2SN*) were analyzed by SDS-PAGE and Western blotting with anti-Syt1 and anti-Syt7 antibodies. Data shown are representative of three independent experiments. *B*, sequence alignment of the polylysine stretch of the C2B of Syt1, -2, and -7 and location of the Lys → Gln mutations in Syt7. Lysines *boxed* and *highlighted* in *red* were shown to be critical for IPP binding in Syt2 (48). *C*, modeling of the wild-type C2B domains of Syt2 and -7 as well as their respective KKK variants. The domains are shown in equivalent orientations with the polylysine region facing the reader. The electrostatic potentials were displayed with Swiss-PdbViewer as *blue* (+7 *kT/e* where *k* is the Boltzmann constant, *T* is absolute temperature, and *e* is the proton charge) and *red* (−7 *kT/e*) (65). *TMD*, transmembrane domain.

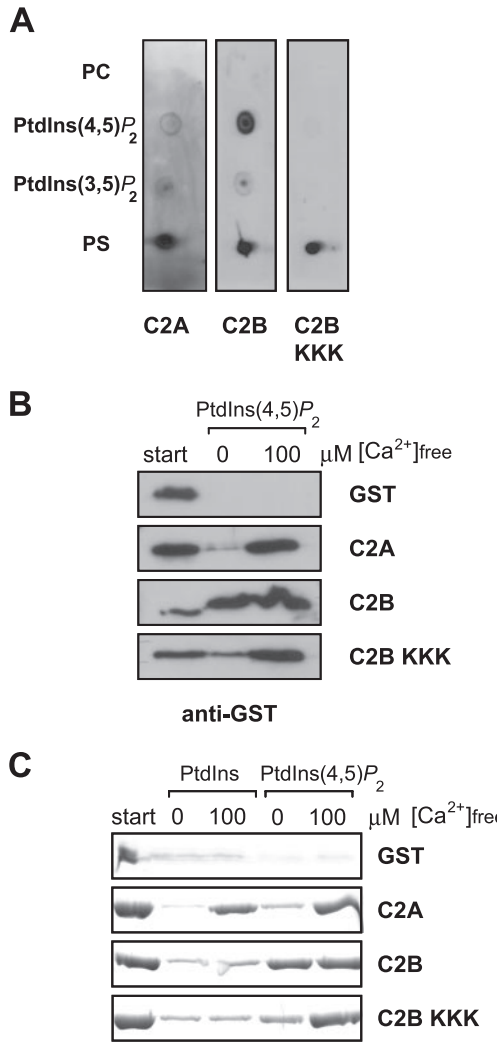


FIG. 5. **Syt7 C2B KKK mutation inhibits calcium-independent PtdIns(4,5)P_2 binding.** *A*, lipid dot-blots (phosphatidylserine (*PS*), phosphatidylcholine (*PC*), PtdIns(4,5)P_2, and PtdIns(3,5)P_2) were incubated with 250 ng/ml recombinant GST or GST-Syt7 C2A, C2B, or C2B KKK mutant fusion proteins. Bound protein was detected by Western blotting using anti-GST antibodies. Data are representative of two independent experiments. *B*, recombinant proteins as in *A* (0.1 μg) were incubated with PtdIns(4,5)P_2 beads in the absence or presence of 100 μM free Ca^{2+}. Proteins bound to the beads were visualized by SDS-PAGE and Western blotting with anti-GST antibodies. Data are representative of three independent experiments. *C*, the GST fusion proteins (5 μg) indicated were used in a phospholipid binding assay in the absence or presence of 100 μM calcium. Bound proteins were analyzed by SDS-PAGE and Coomassie staining. Data are representative of three independent experiments.

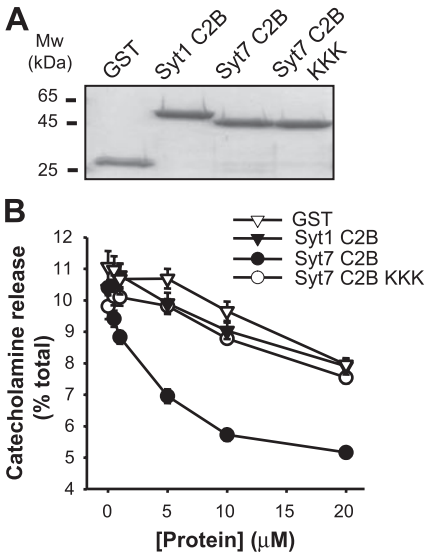


FIG. 6. **Effect of Syt7 C2B KKK mutation on catecholamine secretion.** *A*, recombinant GST fusion proteins were eluted from glutathione beads, and equimolar amounts were visualized by SDS-PAGE and Coomassie staining. *B*, chromaffin cells were permeabilized with 20 μM digitonin in Ca^{2+}- and ATP-free KGEP buffer for 10 min in the absence or presence of increasing concentrations of GST or GST fusion proteins. The supernatant was then removed and replaced by Ca^{2+}- and ATP-containing stimulation KGEP buffer in the continuing presence of recombinant proteins for 5 min. Aliquots were removed and assayed fluorometrically for catecholamine content. Data shown are means ± S.E. (n = 8) and are representative of three independent experiments.

characteristics of this surface (Fig. 4*C*). This suggests that Syt7 may bind directly to PtdIns(4,5)P_2 via this polybasic region. We therefore generated the equivalent Lys → Gln mutations done previously in Syt2 in GST-Syt7 C2B (lysines 320, 321, and 325) to further explore this possibility (Fig. 4*B*, *Syt7 KKK*).

Recombinant GST-Syt7 C2A, C2B, and C2B KKK domains were assessed for PtdIns(4,5)P_2 binding using three different methods: dot-blot overlay, PtdIns(4,5)P_2 bead binding, and liposome binding assays (Fig. 5, *A–C*). Prior to using the proteins in binding assays, the secondary structure content of the wild-type and mutated GST-Syt7 C2B domains was checked by CD spectroscopy, and no significant difference was detected between the two recombinant proteins indicating that the mutant is properly folded (data not shown).

Dot-blot overlay assays (Fig. 5*A*) were carried out, and GST-Syt7 C2A, C2B, and C2B KKK mutant all bound to phosphatidylserine. GST-Syt7 C2B domain also bound strongly to PtdIns(4,5)P_2 but not to PtdIns(3,5)P_2. Mutating the three lysine residues abolished the ability of the GST-Syt7 C2B to interact with PtdIns(4,5)P_2 (Fig. 5*A*). Next recombinant proteins were incubated with PtdIns(4,5)P_2 beads in the presence and absence of calcium, mimicking the chromaffin granule pulldown assay (Fig. 5*B*). In the absence of calcium, Syt7 C2B KKK binding to the PtdIns(4,5)P_2 beads was greatly reduced when compared with that of Syt7 C2B. However, in the presence of calcium, there was no significant difference in PtdIns(4,5)P_2 binding between the Syt7 C2B and Syt7 C2B KKK mutant (Fig. 5*B*). To confirm the PtdIns(4,5)P_2 binding and the effect of the KKK mutation in the context of a lipid bilayer, liposome binding assays were used (Fig. 5*C*). Syt7 C2A bound to the PtdIns(4,5)P_2 liposomes in a calcium-de-

pendent manner in agreement with a previous study (7), although Syt7 C2A also bound PtdIns-containing liposomes in the presence of calcium. Syt7 C2B on the other hand bound PtdIns(4,5)P_2-containing liposomes but not PtdIns-containing liposomes in both the absence and presence of calcium. Syt7 C2B KKK mutant still bound PtdIns(4,5)P_2-containing liposomes in the presence of calcium, but binding in the absence of calcium was greatly reduced (Fig. 5*C* and Supplemental Fig. 1). The addition of physiological concentrations of Mg^{2+} did not significantly affect the binding of Syt7 C2B to PtdIns(4,5)P_2-containing liposomes in either the absence or presence of Ca^{2+} (see Supplemental Fig. 2).

Importance of Synaptotagmin 7 C2B Polybasic Region for Exocytosis—To determine whether the calcium-independent PtdIns(4,5)P_2 binding via the Syt7 C2B polybasic stretch might be important for catecholamine secretion in chromaffin cells, increasing concentrations of standardized GST fusion proteins (Fig. 6*A*) were introduced into digitonin-permeabilized chromaffin cells to measure their effect on catecholamine release (Fig. 6*B*). Although GST alone and GST-Syt1 C2B had little effect on catecholamine secretion as described previously (50), GST-Syt7 C2B inhibited secretion in a concentration-dependent manner and over a concentration range similar to that reported previously in PC12 cells (18). However, over the same concentration range, GST-Syt7 C2B KKK domain had no effect on secretion, suggesting that these three lysine residues are critical for the inhibitory effect of the Syt7 C2B domain (Fig. 6*B*). Together these data point to an important role for the polybasic region of the Syt7 C2B domain in regulating catecholamine secretion from adrenal chromaffin cells.

DISCUSSION

Identification of Chromaffin Granule PtdIns(4,5)P_2-binding Proteins—We used a PtdIns(4,5)P_2 pulldown strategy combined with mass spectrometry to identify PtdIns(4,5)P_2-interacting proteins on chromaffin granules. This method allowed the identification of PtdIns(4,5)P_2-interacting proteins with high specificity as evidenced by the absence of the abundant chromaffin granule proteins chromogranin A and VAMP2 in the PtdIns(4,5)P_2 pulldowns. Our data demonstrate that PtdIns(4,5)P_2 pulldowns can be a powerful technique to identify novel phosphoinositide-binding proteins from specific subcellular compartments. As expected from previous studies, Syt1 was identified. Other proteins isolated were α2/β2 spectrin, α-adaptin A, granuphilin, and Syt7. Peptide mass mapping identified proteins in five bands that were evident by SDS-PAGE of the pulldowns and were not present in the control pulldowns. The identities of two of the proteins, granuphilin and synaptotagmin 1, were also supported by MS/MS analysis. The higher ion scores for peptides from granuphilin and synaptotagmin compared with spectrin and α-adaptin reflect the fact that better peptide extractions are usually achieved from in-gel digests as protein molecular weight decreases. The presence of both spectrin and α-adaptin in the pulldowns was validated by Western blotting.

The interaction between Syt1 and PtdIns(4,5)P_2 is well documented and may play a critical role in allowing Syt1 to bridge the secretory vesicle and plasma membranes during exocytosis. Previous studies have used recombinant proteins (5, 6), and to our knowledge this is the first time binding of native Syt1 to PtdIns(4,5)P_2 has been demonstrated.

Spectrin has been localized previously to chromaffin granules and small synaptic vesicles and may play a role in secretion (41, 51). Both α2 and β2 spectrin contain a PH domain, which has been demonstrated to interact with PtdIns(4,5)P_2 (40). α-Adaptin has also been shown to interact with phosphoinositides including PtdIns(4,5)P_2 (42, 52). Although α-adaptin is involved in endocytosis, it is also present on highly purified synaptic vesicles (44).

Surprisingly another Syt isoform, Syt7, and a synaptotagmin-like protein 4 (granuphilin) were also identified as chromaffin granule PtdIns(4,5)P_2-binding proteins. Both Syt7 and granuphilin have been localized to secretory granules in PC12 cells, although in the case of Syt7 this was only for overexpressed protein (17, 19, 45). This is the first time, to our knowledge, that the two proteins have been identified on chromaffin granules. Granuphilin exists as two isoforms, granuphilin-a and -b (53). Granuphilin-a contains two tandem C2 domains, namely C2A and C2B, whereas granuphilin-b contains only the C2A domain. The detection of a 75-kDa protein by Western blotting and the identification of peptides from the C2B domain of granuphilin by mass spectrometry indicate that we identified granuphilin-a on chromaffin granules. This is the first demonstration that granuphilin-a can bind to PtdIns(4,5)P_2, and it will be interesting to determine the functional importance of this interaction for exocytosis.

A Role for Endogenous Synaptotagmin 7 in Chromaffin Cell Secretory Granule Exocytosis—We showed that in chromaffin cells endogenous Syt7 is present on secretory granules by subcellular fractionation, immunoisolation, and immunocytochemistry, and Syt7 appears to be expressed at much higher levels than in PC12 cells. In PC12 cells, Syt7 is a minor Syt isoform, and endogenous Syt7 has not been detected by immunocytochemistry (18, 19). Previous work on the localization of Syt7 and the effect of Syt7 on secretion in PC12 cells has therefore relied upon overexpression studies, which could have generated the conflicting results regarding plasma membrane *versus* secretory granule localization (17–19). Furthermore the physiological relevance of Syt7 in PC12 cells is unclear because the isoforms important for exocytosis are Syt1 and Syt9 (7, 54). Although overexpressing Syt7 affects regulated exocytosis in PC12 cells (17, 19), small interfering RNA knockdown of endogenous Syt7 expression has little effect on exocytosis of neuropeptide Y from PC12 cells (19), although it may alter the metal sensitivity of release (17). Our data suggest that Syt7 may play a physiological role in the exocytosis of catecholamine-containing secretory vesicles in

chromaffin cells. Syt7 is unlikely to be the major calcium sensor for chromaffin granule exocytosis because in Syt1 knock-out mice the fast component of the exocytic burst (readily releasable pool) is abolished but can be rescued by introduction of Syt1 or Syt2 (55, 56). In chromaffin cells from Syt1 knock-out mice, the slow component (slowly releasable pool) of exocytosis remains; thus Syt7 may function in promoting release of the slowly releasable pool. Alternatively Syt7 may function in release of the readily releasable pool but in a manner dependent on the presence of Syt1 (or the closely related Syt2). Indeed Syt7 and Syt2 have been shown to hetero-oligomerize in the presence of calcium (35), and in the present study we demonstrated that Syt7 and Syt1 can coexist on secretory vesicles.

Significance of the Synaptotagmin 7 C2B Polybasic Region for Function—In PC12 cells, both Syt7 C2A and C2B domains have been shown to inhibit exocytosis (7, 18). In the case of Syt7 C2A, this effect requires the calcium-binding residues. Interestingly the Syt7 C2A binds to both PtdIns(4,5)P_2-containing bilayers and the t-SNARE (target soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex in a calcium-dependent manner (7). We show here that the Syt7 C2A domain bound PtdIns-containing liposomes equally as well as PtdIns(4,5)P_2-containing liposomes in the presence of calcium, whereas the Syt7 C2B specifically bound to PtdIns(4,5)P_2-containing liposomes suggesting a higher level of specificity in the C2B domain with regard to lipid binding.

The inhibitory effect of Syt7 C2B domain on exocytosis in PC12 cells has not, to our knowledge, been explored further even though the C2B may be more important than the C2A for Syt function (57). In the present study we demonstrated that the Syt7 C2B domain inhibits catecholamine release in chromaffin cells. Furthermore we demonstrated that mutating lysines 320, 321, and 325 to glutamine inhibited the calcium-independent PtdIns(4,5)P_2 binding ability of Syt7 C2B and abrogated the inhibitory effect of Syt7 C2B on catecholamine release from permeabilized chromaffin cells. Thus the C2B polylysine stretch is essential for the inhibitory activity of the Syt7 C2B domain. Syt1 C2B has been demonstrated to bind to PtdIns(4,5)P_2 liposomes via two modes. In the calcium-independent mode, the polylysine stretch positions Syt1 alongside the membrane, whereas upon calcium binding, the C2B domain then rapidly inserts into the PtdIns(4,5)P_2-containing bilayer via its calcium-binding loops (5). In this view, Syt7 could also function in a manner analogous to Syt1, contributing to the positioning of Syt7-containing vesicles at sites of high PtdIns(4,5)P_2 on the plasma membrane. Native Syt7 was found to bind PtdIns(4,5)P_2 beads in the absence and to a greater extent in the presence of 100 μM free calcium. This is likely due to the combination of the Syt7 C2A and C2B domains where only the C2B is mediating a calcium-independent binding, whereas both the C2A and C2B domains contribute to calcium-dependent PtdIns(4,5)P_2 binding.

Mutations in the polybasic stretch of Syt1 have a number of important functional consequences including alterations in synaptic vesicle size (58) and reductions in neurotransmitter release (59–61). In *Drosophila*, the polybasic region of Syt1 affects a calcium-independent docking and/or priming step (62). In Syt1 and -2, the polylysine stretch is responsible for a number of interactions in addition to PtdIns(4,5)P_2/IPP binding, including oligomerization and syntaxin/SNAP-25 (Syx/S25) binding (48, 63). In the case of Syt7, oligomerization is independent of the polylysine stretch and instead is mediated by the calcium-binding loops of the C2A and C2B domains cooperatively (35). Syt7 C2AB has been shown to bind to Syx/S25 in the absence of calcium (36), whereas isolated Syt7 C2A domain binds Syx/S25 in the presence of calcium (7). It is unclear whether the polybasic stretch in Syt7 C2B interacts with Syx/S25 and whether it is the calcium-dependent or -independent binding to Syx/S25 that is most important functionally.

A Potential Role for the Synaptotagmin 7-PtdIns(4,5)P_2 Interaction in Other Systems—Increasing evidence supports a role for Syt7 in calcium-dependent lysosome exocytosis that may be important for wound healing (20, 22, 26). Our data indicate that plasma membrane PtdIns(4,5)P_2 could also be important for lysosome exocytosis and membrane resealing. Both the Syt7 C2A and Syt7 C2B inhibit wound healing. Interestingly a recent study established that mutations in Syt7 C2B calcium-binding residues inhibited resealing (26). Although no effect was seen with a Syt7 C2B (K320A,K321A) mutant domain in the initial membrane repair, a reduction in inhibition of facilitation was observed. Thus, the importance of calcium-binding regions and the polylysine stretch of Syt7 C2B may vary between different systems, possibly reflecting a differing importance of C2B effectors. Alternatively the calcium-binding regions and the polylysine stretch may both be important because mutations in both Syt1 C2B polylysine and calcium-binding residues affect evoked neurotransmitter release in *Drosophila* (60, 64). Although further work needs to be done to investigate the relevant contributions of Syt-effector interactions to its function in different systems, our data establish that endogenous Syt7 localizes to secretory granules in adrenal chromaffin cells.

In summary, the strategy of coupling phosphoinositide pull-down with purified chromaffin granules and mass spectrometry analysis allowed the unbiased identification of five major PtdIns(4,5)P_2-binding proteins. These identifications were validated by Western blotting, and the strategy was used for further mechanistic studies. Two novel potential PtdIns(4,5)P_2 effectors for chromaffin granule exocytosis were identified as Syt7 and granuphilin. We have shown here that Syt7 bound PtdIns(4,5)P_2 through both calcium-dependent and -independent interactions and that calcium-independent interactions via the Syt7 C2B domain were important for chromaffin granule exocytosis. This opens a number of possible studies for investigating further the role played by Syt7 and granuphilin in exocytosis. More importantly, such a strategy could also

be used to identify PtdIns(4,5)P_2-interacting proteins from other cellular compartments.

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