The C2B Domain of Rabphilin Directly Interacts with SNAP-25 and Regulates the Docking Step of Dense Core Vesicle Exocytosis in PC12 Cells

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Rabphilin is a membrane trafficking protein on secretory vesicles that consists of an N-terminal Rab-binding domain and C-terminal tandem C2 domains. The N-terminal part of rabphilin has recently been shown to function as an effector domain for both Rab27A and Rab3A in PC12 cells (Fukuda, M., Kanno, E., and Yamamoto, A. (2004) J. Biol. Chem. 279, 13065–13075), but the function of the C2 domains of rabphilin during secretory vesicle exocytosis is largely unknown. In this study we investigated the interaction between rabphilin and SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors, VAMP-2/synaptobrevin-2, syntaxin IA, and SNAP-25) and SNARE-associated proteins (Munc18 IA and Munc13-1) and found that the C2B domain of rabphilin, but not of other Rab27A-binding proteins with tandem C2 domains (i.e. Slp-1, C2B, decreased the number of docked vesicle or fusing at the plasma membrane. These findings suggest that rabphilin is involved in the docking step of regulated exocytosis in PC12 cells, possibly through interaction between the C2B domain and SNAP-25.

Rabphilin was originally identified as a specific GTP-Rab3A-binding protein on secretory granules (1, 2) that is involved in the control of regulated secretion, including neurotransmitter release and hormone secretion (3–6). Recent evidence, however, has indicated that rabphilin modulates SNARE function (8). Thus, it is highly possible that rabphilin physically associates with SNARE proteins itself and/or SNARE-associated proteins but that possibility has never been investigated. In this study we systematically investigated the interaction between rabphilin and SNAREs and SNARE-associated proteins by co-immunoprecipitation assays and found that the C2B domain of rabphilin directly interacts with isolated SNAP-25, a target SNAP localized on the plasma membrane. Because there has never been a detailed description of the function of the C2B domain of rabphilin in the recruitment, docking, priming, and fusion of secretory vesicles in live cells, we also investigated the function of the C2B domain of rabphilin on the motion of a single dense core vesicle during exocytosis in PC12 cells by total internal reflection fluorescence microscopy (TIRF, also called evanescent wave or evanescent microscopy) (27) using vesicle-targeted fluorescent proteins (28–31). Expression of the rabphilin-ΔC2B mutant lacking SNAP-25 binding activity inhibited vesicle docking and markedly decreased the number of exocytotic events without affecting the kinetics of vesicle fusion. Based on our findings, we discuss
SNAP-25 Interaction with the C2B Domain of Rabphilin

the possible function of the C2B domain of rabphilin in docking of dense core vesicles to the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials—Anti-Rab27A, anti-Munc18, and anti-Munc13 mouse monoclonal antibodies were obtained from BD Transduction Laboratories (Lexington, KY). Anti-VAMP-2 and anti-synaptotagmin (Syt) I/SYA148 mouse monoclonal antibodies were from StressGen Biotechnologies (Victoria, British Columbia, Canada). Anti-T7 tag antibody-conjugated agaroose and horseradish peroxidase (HRP)-conjugated anti-T7 tag mouse monoclonal antibody were from Merrck Biosciences Novagen. Anti-syntaxin I/HPC-1 mouse monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 affinity gel, HRP-conjugated anti-FLAG M2 mouse monoclonal antibody, HRP-conjugated anti-HA tag mouse monoclonal antibody, and anti-SNAP-25 mouse monoclonal antibody were from Sigma. Anti-Rab27B rabbit polyclonal antibody was prepared as described previously (24).

Plasmid Construction—pEF-T7-GST-rabphilin, pEF-T7-rabphilin-ΔRB, pEF-T7-rabphilin-C2A, pEF-T7-rabphilin-C2B, and pEF-T7-rabphilin-ΔC2B were constructed by conventional PCR techniques as described previously (18, 32, 33), and all constructs were verified by DNA sequencing. The sequences of the oligonucleotides used are available from the authors upon request. Rabphilin-ΔRB contains amino acid residues 187–681 of mouse rabphilin, rabphilin-C2A contains amino acid residues 376–507 of mouse rabphilin, rabphilin-C2B, amino acid residues 528–681 of mouse rabphilin, and rabphilin-ΔC2B, amino acid residues 1–507 of mouse rabphilin. The rabphilin and rabphilin-ΔC2B fragments were also subcloned into the pmRFP-C1-gk vector modified from pmRFP-C1 (Clontech) by introducing a short Gly linker (GGSSGTTGGS) just downstream of monomer red fluorescent protein (mRFP). Plasmid encoding neuropeptide Y (NPY)-Venus (pVenus-N1-NPY) was generously provided by Atsushi Miyawaki (34). Other mammalian expression vectors (pEF-FLAG-syntaxin IA, pEF-FLAG-SNAP-25, pEF-HA-Rab27A, pEF-FLAG-VAMP-2, pEF-FLAG-Munc18-1, pEF-T7-rabphilin, pEF-T7-rabphilin (E50A/I54A), pEF-T7-GST-Noc2, and pEF-T7-Slp1-5) were prepared as described previously (11, 13, 23, 35, 36). The SNAP-25 fragment was also subcloned into the BamHI/NotI site of the pGEX-4T-3 vector (Amershams Biosciences).

Pull-down Assay—PC12 cell culture and transfection of plasmids into PC12 cells were performed as described previously (22, 37). Three days after transfection, PC12 cells (one 6-cm dish) expressing T7-GST-rabphilin, T7-GST-Noc2, or T7-GST as a control, were harvested and homogenized in 1 ml of 50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 1 mM MgCl2, 0.5 mM GTPyS, and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, and 10 mM pepstatin A) in a glass-Teflon Potter homogenizer by 10 strokes at 900–1000 rpm, and the proteins were solubilized with 1% Triton X-100 at 4 °C for 1 h. After solubilization with 1% Triton X-100, insoluble material was removed by centrifugation at 15,000 rpm for 10 min, and the GST fusion proteins expressed were affinity-purified on glutathione-Sepharose beads (wet volume 20 μl; Amershams Biosciences) according to the manufacturer’s instructions. After washing the beads five times with 50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 1 mM MgCl2, and 0.2% Triton X-100, proteins bound to the beads were analyzed by 12.5% SDS-PAGE followed by immunoblotting with anti-syntaxin I (1/1000 dilution), anti-SNAP-25 (1/1000 dilution), anti-VAMP-2 (1/1000 dilution), anti-Munc18-1 (1/250 dilution), anti-Munc13 (1/250 dilution), anti-Syt I (1/250 dilution), anti-Rab27A (1/250 dilution), anti-Rab27B (2 μg/ml), and HRP-conjugated anti-T7 tag antibody (1/10,000 dilution), respectively, as described previously (23, 33, 38). The immunoreactive bands were visualized by means of enhanced chemiluminescence (Amershams Biosciences).

Co-immunoprecipitation Assay in COS-7 Cells—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μM streptomycin, at 37 °C under 5% CO2, pEF-T7, pEF-FLAG, and/or pEF-HA vectors (total of 4 μg of plasmids) were transfected into COS-7 cells (7.5 × 106 cells, the day before transfection/10-cm dish) by using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions. Three days after transfection, cells were harvested and homogenized, and total cell lysates were prepared as described previously (37). The total cell lysates (400 μl) were incubated with anti-T7 tag antibody-conjugated agarose beads (wet volume 20 μl) with gentle agitation at 4 °C for 1 h, and the proteins bound to the beads were analyzed by 10% (or 12.5%) SDS-PAGE followed by immunoblotting with HRP-conjugated anti-T7 tag antibody (1/10,000 dilution), HRP-conjugated anti-FLAG M2 antibody (1/10,000 dilution), and/or HRP-conjugated anti-HA tag antibody (1/10,000 dilution) as described previously (33, 38). The blots shown in this paper are representative of at least two independent experiments.

Direct Interaction between T7-rabphilin and GST-SNAP-25—The T7-rabphilin beads (wet volume, 10 μl) were prepared as described above. GST-SNAP-25 was expressed in bacteria and purified with glutathione-Sepharose beads by standard protocols (18, 39). The concentration of GST-SNAP-25 was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories), using bovine serum albumin as a reference. The T7-rabphilin beads were incubated for 1 h at 4 °C with 400 μl of various concentrations of GST-SNAP-25 (0.02–5 μM) in 50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 1 mM MgCl2, 0.2% Triton X-100, and 2 mM EGTA (or 0.5 mM CaCl2), and proteins bound to the beads were analyzed by 10% SDS-PAGE followed by staining with Coomassie Brilliant Blue R-250. The intensity of the bands on the gel was captured and quantified with Lane Analyzer software (version 3.0) (ATTO Corp., Tokyo, Japan). All statistical analyses and curve fitting were performed with a GraphPad Prism computer program (version 4.0, GraphPad Software, San Diego, CA).

TIRF Microscopy—PC12 cells were cultured as described above (37). For TIRF imaging, PC12 cells were plated onto poly-l-lysine-coated coverslips, and cells were co-transfected with 2 μg of pVenus-N1-NPY, and 2 μg of pmRFP-C1, pmRFP-C1-gk-rabphilin, or pmRFP-C1-gk-rabphilin-ΔC2B by using Lipofectamine 2000 according to the manufacturer’s instructions. The imaging was performed in a modified Ringer buffer at 37 °C (130 mM NaCl, 3 mM KCl, 5 mM CaCl2, 1.5 mM MgCl2, 10 mM glucose, and 10 mM HEPES, pH 7.4). Stimulation with high KCl was
FIGURE 1. Rabphilin is copurified with SNAP-25 and Rab27 but not with other SNAREs or SNARE-associated proteins. A, rabphilin, but not Noc2, interacts with SNAP-25 in PC12 cells. The GST pull-down assay was performed as described under “Experimental Procedures.” The proteins that bound to the beads coupled with GST alone (lane 1), GST-rabphilin (lane 2), or GST-Noc2 (lane 3) were analyzed by 12.5% SDS-PAGE followed by immunoblotting with anti-syntaxin I, anti-SNAP-25, anti-VAMP-2, anti-Munc18, anti-Munc13, anti-Syt I, anti-Rab27A, and HRP-conjugated anti-T7 tag antibody. B, rabphilin interacts with SNAP-25 in mouse brain. Agarose beads coupled with T7-rabphilin (lane 2) or beads alone as a negative control were incubated with the solubilized membrane fraction of mouse cerebellum, and proteins bound to the beads were analyzed by 12.5% SDS-PAGE followed by immunoblotting with the antibodies indicated as described above. Note that rabphilin interacted with SNAP-25 (closed arrowheads) and Rab27 (open arrowheads). The asterisk in B indicates the heavy chain of IgG. Input means 1/80 volume of the reaction mixture used for immunoprecipitation (lane 4 in A and lane 3 in B). The positions of the molecular mass markers (×10−3) are shown on the left.

RESULTS

Co-purification of SNAP-25 and Rab27A with Rabphilin from PC12 Cell Lysates—To investigate the possible interaction between rabphilin and SNAREs, i.e. syntaxin I, SNAP-25, and VAMP-2, or SNARE-associated proteins, i.e. Munc13 and Munc18, GST-tagged rabphilin was transiently expressed in PC12 cells, and GST-rabphilin and rabphilin-interacting molecules were affinity-purified on glutathione-Sepharose beads (see ‘Experimental Procedures’ for details). Immunoblot analyses showed that both SNAP-25 and Rab27A were co-purified with GST-rabphilin (closed and open arrowheads, respectively, lane 2 in Fig. 1A), but no syntaxin I, VAMP-2, Munc13, Munc18, or Syt I was detected. By contrast, SNAP-25 was not co-purified with GST alone (Fig. 1A, lane 1), GST-Noc2, another Rab27A-binding protein without tandem C2 domains in PC12 cells (13, 40) (Fig. 1A, lane 3), or GST-Slp4-a (23, 25, 26) (see also supplemental Fig. 1), although Noc2 and Slp4-a did interact with Rab27A. Similarly, both SNAP-25 and Rab27B (a closely related isoform of Rab27A expressed in brain) (41) were co-purified with T7-rabphilin from the solubilized membrane fraction of mouse cerebellum (Fig. 1B, lane 2).

Interaction between rabphilin and SNAP-25 was further investigated by co-immunoprecipitation assay using COS-7 cells, which do not endogenously express either rabphilin or neuronal SNAREs (or SNARE-associated proteins) (35). In brief, agarose beads coupled with T7-rabphilin were incubated with COS-7 cell lysates containing FLAG-syntaxin IA, FLAG-SNAP-25, FLAG-VAMP-2, or FLAG-Munc18-1, in the presence and absence of HA-Rab27A, and FLAG-tagged proteins and HA-Rab27A that had bound to the beads were detected by immu-
nblotting with the specific antibodies indicated (Fig. 2). As expected, rabphilin specifically interacted with SNAP-25, but not with other SNAREs or Munc18-1, irrespective of the presence of Rab27A (Fig. 2, third panel, lanes 2 and 6). It should be noted that SNAP-25 did not interact with Slp1-5 (11, 14, 36), another Rab27A-binding protein with tandem C2 domains at the C terminus (supplemental Fig. 1), suggesting that rabphilin is the only Rab27A-binding protein that physically associates with SNAP-25.

The C2B Domain of Rabphilin Functions as a SNAP-25-binding Site

Next, we attempted to map the minimal SNAP-25-binding site in rabphilin. To do so, we prepared three truncated mutants of rabphilin, i.e. the RBD, C2A domain, and C2B domain, and tested their SNAP-25 binding activity by co-immunoprecipitation assay using COS-7 cells. As shown in Fig. 3A, the C2B domain interacted with SNAP-25, the same as the full-length protein (third panel, lanes 1 and 4), but the C2A domain and the RBD did not. Similarly, the rabphilin-ΔC2B mutant did not interact with SNAP-25, but the rabphilin (E50A/I54A) mutant, which lacks Rab27A binding activity (13), did (Fig. 3B, third panel, lanes 2–4). The direct interaction between rabphilin and SNAP-25 was further investigated by using purified components, i.e. T7-rabphilin and GST-SNAP-25. Interaction between rabphilin and SNAP-25 was also observed when the purified proteins were used, even in the presence of 2 mM EGTA (Fig. 4A, closed arrowhead), and 0.5 mM Ca2+ slightly increased the SNAP-25 binding activity (Fig. 4B, closed arrowhead). A calculation of the EC50 values by analysis of the dose dependence curve of GST-SNAP-25 (Fig. 4C) yielded 0.817 and 0.405 μM for the interaction between rabphilin and SNAP-25 in the absence and presence, respectively, of Ca2+, whereas the Bmax values were unchanged (1.06 mol of GST-SNAP-25/mol of T7-rabphilin). These findings indicate

**SNAP-25 Interaction with the C2B Domain of Rabphilin**
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Figure 4. Direct interaction between rabphilin and SNAP-25. A, Ca²⁺-independent interaction between purified T7-rabphilin and GST-SNAP-25. T7-rabphilin beads were incubated with the various concentrations of GST-SNAP-25 indicated, and bound GST-SNAP-25 was visualized by Coomassie Brilliant Blue (CBB) R-250 staining as described under “Experimental Procedures.” Red open arrowheads indicate the positions of T7-rabphilin and GST-SNAP-25, respectively. The asterisks indicate the degradation products of T7-rabphilin. The positions of the molecular mass markers (× 10⁻⁵) are shown on the left. The results shown are representative of three independent experiments. C, dose dependence curve of GST-SNAP-25 in the presence and absence of Ca²⁺; T7-rabphilin and GST-SNAP-25 bands on polyacrylamide gels (in A and B) were captured and quantified as described under “Experimental Procedures.” The EC₅₀ values of 0.817 µM for the rabphilin-SNAP-25 interaction in the presence of 2 mM EGTA (open circles) and 0.405 µM for the rabphilin-SNAP-25 interaction in the presence of 0.5 mM Ca²⁺ (closed circles) were calculated with GraphPad Prism software (version 4.0). Bars indicate the S.E. of three independent experiments. D, formation of the tripartite protein complex of Rab27A, rabphilin, and SNAP-25. T7-rabphilin beads were first incubated with the COS-7 cell lysates transfected with pEF-BOS alone (lane 1) or pEF-HA-Rab27A (lane 2) in 50 mM HEPES-KOH, pH 7.2, 150 mM NaCl, 1 mM MgCl₂, 0.5 mM GTP-γ-S, and 0.2% Triton X-100. After extensively washing the beads with the binding buffer, the beads were then incubated with 5 µM GST-SNAP-25, and bound proteins were visualized by Coomassie Brilliant Blue R-250 staining. Note that T7-rabphilin (open arrowhead) simultaneously interacted with GST-SNAP-25 (closed arrowhead) and HA-Rab27A (arrow). The asterisks indicate the degradation products of T7-rabphilin. The positions of the molecular mass markers (× 10⁻⁵) are shown on the left. The results shown are representative of two independent experiments.

Figure 5. Effect of rabphilin expression on the number of docked vesicles to the plasma membrane in PC12 cells. A, confocal images of a PC12 cell showing the distribution of NPY-Venus (left) and mRFP-rabphilin or mRFP-rabphilin-ΔRBD (middle). Right panels indicate the overlay between NPY-Venus and mRFP-rabphilin or mRFP-rabphilin-ΔRBD. B, typical TIRF images of plasma membrane-docked vesicles observed before high KCl stimulation in control cells (left), mRFP-rabphilin-expressing (middle) cells, and mRFP-rabphilin-ΔC2B-expressing cells (right). Insets are 6-fold magnified images of the boxed area. Scale bars in A and B, 5 µm. C, the density of docked vesicles before and after stimulation was determined by counting the vesicles in each image (n = 7 cells in each). D, the number of NPY-Venus spot disappearance events in 5 min was counted as fusion events (n = 7 cells each). Data shown are mean values ± S.E. * p < 0.05 and *** p < 0.001, respectively, in comparison with the control.

that 1 molecule of rabphilin binds 1 molecule of SNAP-25 via the C2B domain and that Ca²⁺ promotes SNAP-25 binding to rabphilin because of the 2-fold increase in affinity. Because SNAP-25 and Rab27A interact with the distinct domains of rabphilin, a tripartite protein complex of Rab27A, rabphilin, and SNAP-25 can be formed (Fig. 4D, lane 2).

Rabphilin Promotes Dense Core Vesicle Exocytosis by Increasing the Number of Docked Vesicles to the Plasma Membrane in PC12 Cells—Although expression of rabphilin in certain endocrine cells has been shown to promote dense core vesicle exocytosis (3, 4, 13), the step or steps in vesicle exocytosis, i.e. recruitment, docking, and fusion, that are regulated by rabphilin have never been identified. To resolve this matter, we co-expressed rabphilin and NPY-Venus, which is specifically targeted to dense core vesicles (30, 34), in PC12 cells and monitored the fate of NPY-Venus during and after individual exocytotic events near the plasma membrane by TIRF microscopy. First, we investigated colocalization between mRFP-rabphilin and NPY-Venus in resting PC12 cells by confocal microscopy, and as expected, most NPY-Venus-positive vesicles were found to be colocalized with mRFP-rabphilin-positive vesicles (Fig. 5A, top panels), consistent with...
SNAP-25 Interaction with the C2B Domain of Rabphilin

To determine whether mRFP-rabphilin regulates vesicle transport, docking to the plasma membrane, or fusion in live PC12 cells, we counted the number of plasma membrane-associated vesicles before and after high KCl stimulation (70 mM) by TIRF microscopy (Fig. 5B and supplemental movies 1–3). As shown in Fig. 5C, the numbers of plasma membrane-docked vesicles both before and after stimulation were significantly higher in the mRFP-rabphilin-expressing cells than in the control cells that expressed mRFP alone. The total number of NPY-Venus release events from cells expressing mRFP-rabphilin was also markedly higher than in the control cells (Fig. 5D, gray bar). Exocytotic events were also detected much less frequently in mRFP-rabphilin-C2B-expressing cells than in mRFP-rabphilin-expressing cells, or even in the control cells (Fig. 5D), although the kinetics of individual fusion events were identical in each case (Fig. 6). Taken together, these findings suggest that the C2B domain of rabphilin regulates the docking step of dense core vesicles to the plasma membrane rather than the dense core vesicle fusion step.

DISCUSSION

Although a previous genetic analysis of C. elegans rabphilin-SNARE double mutants suggested that rabphilin modulates SNARE function (8), rabphilin-binding proteins identified thus far (e.g. α-actinin, β-adenilin, GTP cyclohydrolase I, Rabaptin5, and annexin A4) (43–48) have no relation to the function of neuronal SNAREs or SNARE-related proteins, and a physical association between rabphilin and SNAREs had never been elucidated even in vitro. In the present study we obtained for the first time biochemical evidence that the C2B domain of rabphilin is necessary and sufficient for direct Ca2+-independent interaction with SNAP-25 but not with syntaxin IA or VAMP-2 (Figs. 1–4). We also demonstrated by TIRF microscopy that expression of rabphilin significantly increases the number of docked dense core vesicles to the plasma membrane in PC12 cells (Fig. 5) without altering the kinetics of individual exocytotic events (Fig. 6), whereas expression of a rabphilin-C2B mutant lacking SNAP-25 binding activity significantly decreased the number of high KCl-induced NPY-Venus release events and the number of plasma membrane-docked vesicles after high KCl stimulation (Fig. 5). The most straightforward explanation for this result is that rabphilin promotes docking of dense core vesicles to the plasma membrane by linking the Rab27A on the vesicle via the RBD and SNAP-25 at the plasma membrane via the C2B domain. Very recently, another Rab27A effector, Slp2-a in melanocytes, has been shown to be involved in the anchoring of melanosomes to the plasma membrane through simultaneous interaction with Rab27A on the melanosome and phosphatidylinerine in the plasma membrane via the C2A domain (49), and Slp4-a/granuphilin-a has been shown to be involved in the docking of insulin-containing vesicles to the plasma membrane through interaction with Rab27A on the vesicle and syntaxin IA/Munc18-1 at the plasma membrane in pancreatic β-cell lines (26, 50).

It is interesting that neither melanocytes nor pancreatic β-cells endogenously express rabphilin, and thus it is highly possible that the general function of Rab27A effectors with tandem C2 domains (i.e. Slp1-5 and rabphilin) may be the docking of Rab27A-bound organelles to the plasma membrane with "two hands." These Rab27A effectors seize specific organelles with Rab27A by the N-terminal RBD (one hand) and directly or indirectly tether them to the plasma membrane by the C-terminal tandem C2 domains (the other hand). Because the biochemical properties of the tandem C2 domains of Slp1-5 and rabphilin differ in terms of phospholipid and protein interactions (14, 23, 25, 36, 51–53) and these Rab27A effectors are differentially expressed in mouse tissues (1, 36, 42, 51), the docking mechanisms of these Rab27A effectors may be different, because Slp1-5 did not interact with SNAP-25 at all (sup-
SNAP-25 Interaction with the C2B Domain of Rabphilin

Supplemental Fig. 1). and rabphilin may be the only Rab27A effector that utilizes SNAP-25 for docking process in certain neuroendocrine cells (e.g. chromaffin cells and PC12 cells).

Because the rabphilin-ΔC2B mutant used in this study completely lacked the C2B domain, we cannot rule out the possibility that unidentified C2B ligands other than SNAP-25 regulate the docking process of dense core vesicle exocytosis in PC12 cells. One of the candidate ligands is phosphatidylinositol 4,5-bisphosphate in the plasma membrane (19). However, because phosphatidylinositol 4,5-bisphosphate interacts with SNAP-25 at the plasma membrane via the C2B domain.

The docking step of regulated exocytosis in PC12 cells, possibly through interaction with Rab27A on the vesicle via the RBD and with t-SNAREs, is important for promotion of dense core vesicle exocytosis in PC12 cells, specifically the docking step to the plasma membrane. Based on our findings, we hypothesize that the C2B domain of rabphilin plays an essential role in the docking step of regulated exocytosis in PC12 cells, possibly through interaction with Rab27A on the vesicle via the RBD and with t-SNARE SNAP-25 at the plasma membrane via the C2B domain.

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