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Rab3A is a new interacting partner of synaptotagmin I and may modulate synaptic membrane fusion through a competitive mechanism

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ABSTRACT

Rab3 and synaptotagmin have been reported to be the key proteins that have opposite actions but cooperatively play critical regulatory roles in selecting and limiting the number of vesicles released at central synapses. However, the exact mechanism has not been fully understood. In this study, Rab3A and synaptotagmin I, the most abundant isoforms of Rab3 and synaptotagmin, respectively, in brain were for the first time demonstrated to directly interact with each other in a Ca^{2+}-independent manner, and the KKKK motif in the C2B domain of synaptotagmin I was a key site for the Rab3A binding, which was further confirmed by the competitive inhibition of inositol hexakisphosphate. Further studies demonstrated that Rab3A competitively affected the synaptotagmin I interaction with syntaxin 1B that was involved in membrane fusion during the synaptic vesicle exocytosis. These data indicate that Rab3A is a new synaptotagmin I interacting partner and may participate in the regulation of synaptic membrane fusion and thus the vesicle exocytosis by competitively modulating the interaction of synaptotagmin with syntaxin of the t-SNARE complex in presynaptic membranes.

1. Introduction

Neurotransmitter release is mediated by Ca^{2+}-triggered exocytosis of synaptic vesicles upon arrival of an action potential at the presynaptic active zone of nerve terminals [1,2]. Protein–protein interactions in a synapse represent the key events for the correct targeting of the vesicles and for the precise spatio-temporal timing of exocytosis. It has been shown that the synaptic core complex is formed by three proteins: synaptobrevin, syntaxin and SNAP-25. Specific interactions between t-SNAREs (such as syntaxin 1 and SNAP-25 in the presynaptic membrane) and v-SNAREs (such as VAMP/synaptobrevin in the synaptic vesicle membrane) are critical for synaptic vesicle exocytosis [3–5]. Many other proteins, including complexin, Munc-18, synaptophysin and tomosyn, can interact with the SNAREs and presumably regulate the formation or disassembly of this complex [6–9]. Although a great deal of effort has been made to probe into the molecular mechanism of neurotransmission, how the process is regulated precisely is still not completely understood. There are many unknown interactions between different proteins involved in the process to be identified.

Recent progress in blue native polyacrylamide gel electrophoresis (BN-PAGE) has allowed the characterization of protein–protein interactions more directly and efficiently than before. It offers a unique advantage of separating native protein complexes present in a membrane proteome or cell lysate sample without dissociating them. When combined with HPLC and mass spectrometry, it can identify almost all the components of a protein complex and thus discovers new protein interactions as well as the new interacting partners of a particular protein. In the present work, we employed BN-PAGE and CapLC–MS/MS to analyze the protein complexes in synaptosomes isolated from rat brain and identified several protein complexes as well as their components including those involved in vesicle trafficking and exocytosis. Of the identified proteins, Rab3A and synaptotagmin I, two important proteins mediating synaptic vesicle exocytosis [10], were found to exist in the same complex and other strategies including co-immunoprecipitation, GST pull-down assay, site-directed mutagenesis, Western blotting and competitive inhibition analysis were employed to further validate the

Abbreviations: BN-PAGE, blue native polyacrylamide gel electrophoresis; GST-Syt I CR, GST-fused cytoplasmic region of synaptototagmin I; GST-C2A, GST-fused C2A domain; GST-C2B, GST-fused C2B domain; IP_6, inositol 1,2,3,4,5,6-hexakisphosphate; SNARE, SNAP (soluble NSF attachment protein) receptor.

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protein interaction and to probe into its molecular mechanism. The results suggest that Rab3A is a novel synaptotagmin I interacting partner and can compete with syntaxin 1 of t-SNARE complex for binding to the C2B domain of synaptotagmin I, thereby regulating the membrane fusion and thus the synaptic vesicle exocytosis.

2. Materials and methods

2.1. Materials

Plasmid pcDNA3.1 was purchased from Clontech. pGEX-4T-1 was from Pharmacia. Protein-A agarose beads were from Santa Cruz. Anti-Rab3A, anti-syntaxin 1B, anti-synaptotagmin I and the peroxidase-conjugated secondary antibodies were from Abcam (Cambridge, UK). Adult Sprague–Dawley rats (weighting 200–250 g) were purchased from the Center South University (Changsha, China). All the experimental procedures involving animals were conducted according to the requirements of the Provisions and General Recommendations of Chinese Experimental Animal Administration Legislation. All rats were allowed food and water ad libitum until the time of death.

2.2. Synaptosome isolation and BN-PAGE of synaptic protein complexes

For sample preparation, animals were executed by cervical dislocation after anesthetized with ethyl ether, and the brains were acutely dissected on ice, from which the synaptosomes were isolated essentially as described previously [11]. To solubilize and extract synaptic protein complexes, synaptosome sample was vigorously pipetted in a buffer (50 mM NaCl, 50 mM imidazole, 2 mM 6-aminohexanoic acid, 1 mM EDTA, pH 7.0) containing 2% Triton X-100, followed by centrifugation at 18,000*g* for 20 min at 4 °C. The supernatant was recovered and its protein content was determined using a Bio-Rad DC protein assay kit (Bio-Rad). BN-PAGE was performed according to the published protocols [12,13]. Protein complexes were separated on a 4–10% gradient separation gel with a 3.5% stacking gel. The cathode buffer (7.5 mM imidazole, 50 mM tricine) containing 0.02% (w/v) Coomassie Brilliant Blue G250 and the anode buffer (25 mM imidazole/HCl, pH 7.0) were chilled to 4 °C before use. Electrophoresis was begun at 100 V at 4 °C. After about 1 h, the cathode buffer was replaced by the same buffer containing 0.002% of G250, and the electrophoresis was continued at voltage of 200 V at 4 °C until stop. After completion of the electrophoresis, the BN-PAGE gel was fixed and then stained with Coomassie Brilliant Blue G250.

2.3. In-gel digestion and CapLC-MS/MS analysis

The proteins in individual bands manually excised from the gels after BN-PAGE were subjected to trypsin digestion and analyzed by capilary column liquid chramatography–tandem mass spectrometry (CapLC–MS/MS). The in-gel digestion, mass spectrometric analysis, data processing and bioinformatic analysis were performed according to the methods described previously [14].

2.4. Rab3A and synaptotagmin I co-immunoprecipitation from rat brain synaptosome extract

The isolated rat brain synaptosomes were homogenized in a buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA, pH 7.4) containing 2% Triton X-100 and then centrifuged at 18,000*g* for 15 min at 4 °C. The supernatant was pre-cleaned with Protein A agarose for 10 min on ice. An aliquot of synaptosome extract (about 1 mg of proteins) was incubated overnight at 4 °C with 15 μg of anti-Rab3A (Abcam, UK), anti-synaptotagmin I (Abcam, UK) or nonspecific control IgGs (Abcam Biotechnology), respectively. For determining the effect of Ca^{2+} on the immunoprecipitation, the buffers that contained 2 mM EGTA or 150 μM $CaCl_2$, respectively, were used in the binding experiments. Protein A agarose beads were added and the reaction mixture was incubated overnight at 4 °C. After the beads were sequentially washed by a low-salt buffer (150 mM NaCl, 50 mM Tris, 1 mM EDTA and 1% Triton X-100, pH 7.4) and a high-salt buffer (300 mM NaCl, 50 mM Tris, 1 mM EDTA and 1% Triton X-100, pH 7.4), 2 × SDS loading buffer was added and the solution was incubated for 10 min at 65 °C. After centrifugation, the proteins that were eluted from the beads were recovered and then analyzed by SDS–PAGE. The separated proteins in parallel lanes were subjected to Western blotting and CapLC–MS/MS analysis, respectively.

2.5. Construction and expression of fusion proteins

RT-PCR of rat brain mRNA was used to produce glutathione S-transferase (GST)-fused proteins in pGEX-4T-1 (Pharmacia) [15], including GST-Syt I CR (the cytoplasmic region of synaptotagmin I, residues 96–421), GST-C2A (C2A domain, residues 128–269), GST-C2A mutant (C2A domain with a mutated polylysine motif K189A/K190A/K191A/K192A), GST-C2B (C2B domain, residues 262–385), GST-C2B mutant (C2B domain with a mutated polylysine motif K324A/K325A/K3266A/K327A). All the recombinant proteins were expressed in *Escherichia coli* BL21 cells, a protease-deficient strain (Novagen), and were purified according to the standard procedures [15]. His_6-tagged fusion proteins including His_6-syntaxin 1B and His_6-Rab3A were expressed as described [16]. The His_6-tagged fusion proteins were purified using a Ni^{2+}-charged nitrilotriacetic acid agarose columns (Qiagen, Chatsworth, CA), eluted with 500 mM imidazole in PBS. The eluates were concentrated with Centriprep-10 filtration units (Amicon) and dialyzed in a 10,000 MW cut off dialysis cassette (Pierce) against PBS containing 0.1% Triton X-100 and 0.1% glycerol.

2.6. GST pull down assay

GST fusion proteins, including GST-fused cytoplasmic region of synaptotagmin I, C2A, C2B and two domain mutants, as well as full-length GST alone were purified using Glutathione-Sepharose beads. Bead-bound recombinant proteins were separately incubated overnight at 4 °C with synaptosome extract in a HNa buffer (10 mM Hepes-NaOH, 150 mM NaCl, 1 μM pepstatin A, 2 μM leupeptin, 0.3 mM phenylmethylsulfonyl fluoride, pH7.4) containing 0.5% Triton X-100. After incubation, the beads were washed 3 times with 1 ml of the HNa buffer containing 0.1% Triton X-100. The bound proteins were eluted with the 2 × SDS loading buffer and subjected to SDS–PAGE and Western blotting analyses. For detecting the effect of Ca^{2+} on the pull down, buffers containing 2 mM EGTA or 150 μM $CaCl_2$ were used and compared. To determine the effect of inositol hexakisphosphate (IP_6) on the interaction between Rab3A and synaptotagmin I, a buffer containing 100 μM IP_6 was used.

2.7. Competition analysis of Rab3A and syntaxin 1B binding to the C2B domain of synaptotagmin I

For investigating the effect of Rab3A on the interaction between synaptatogmin I and syntaxin, a main component of t-SANRE that mediates the fusion of vesicle with presynaptic membrane, Glutathione-Sepharose bead-bound GST-C2B (5 μg) and a constant amount of the purified recombinant His_6-Rab3A (3 μg) were incubated with increasing amounts of purified recombinant His_6-syntaxin 1B (0–1.25 μM final concentration) in 1 ml of TBS buffer

containing 0.1% Triton X-100 for 4–5 h at 4 °C under constant agitation. Then the beads were collected by centrifugation at 10,000g for 10 min and washed 4 times with 1 ml of TBS buffer containing 0.1% Triton X-100. Finally, the bound proteins were eluted from the beads and analyzed by Western blotting. Conversely, the possible competition of Rab3A and syntaxin in binding to C2B domain was investigated by incubating a constant amounts of Glutathione-Sepharose bead-bound GST-C2B (5 μg) and syntaxin 1B (16 μg) with different amounts of Rab3A (0–2.5 μM final concentration) in a similar way as above.

3. Results

3.1. Potential interaction between Rab3A and synaptotagmin I suggested by protein complex analysis

Blue native PAGE was employed to separate the protein complexes in the mild detergent extract of rat synaptosomes. Fig. 1A shows a representative BN-PAGE image of the protein sample (100 μg of proteins) solubilized with Triton X-100 from synaptosomes. The gradient gel of 4–10% acrylamide resolved 6 main protein complex bands, whose molecular masses were in the range of 66–800 kDa. The protein components in each complex were identified with CapLC–MS/MS analysis combined with protein database searching. When the proteins in band 4 were identified, it was found that Rab3A and synaptotagmin I, two proteins playing critical regulatory roles in synaptic vesicle exocytosis [10], existed in the same complex, suggesting that there might be interaction between them. In addition, more than 20 other proteins, isoforms or subunits that were closely related to the functions of synaptosomes were also identified in the complex, such as synaptoporin, syntaxin 1B and synaptophysin (Supplementary Table S1).

3.2. Validation of Rab3A interaction with synaptotagmin I by co-immunoprecipitation

To validate the potential interaction between Rab3A and synaptotagmin I, co-immunoprecipitation from synaptosome extract was performed with antibodies specific for Rab3A and synaptotagmin I, respectively. After the immunoprecipites obtained with the Rab3A-specific antibody or preimmunization immunoglobulins G (IgGs) were resolved by SDS–PAGE and transferred onto a PVDF membrane (GE Healthcare), Western blots were probed with a specific primary antibody against synaptotagmin I and an HRP-conjugated secondary antibody sequentially. The results (Fig. 1B, left) showed that synaptotagmin I was co-immunoprecipited by the Rab3A-specific antibody but not by the control IgG pools. Conversely, Rab3A protein was confirmed to be specifically retained by the synaptotagmin I-specific antibody (Fig. 1B, right). These results further validated that Rab3A and synaptotagmin I were co-assembled into a protein complex.

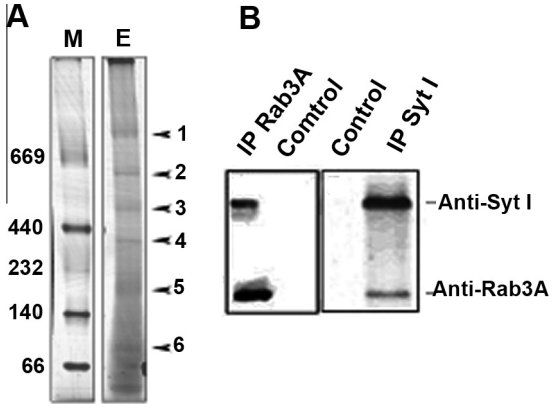

Fig. 1. Blue native PAGE and Western blotting showing the potential interaction between Rab3A and synaptotagmin I. (A) Blue native PAGE of rat brain synaptosome extract. The gel bands used for trypsinolysis and CapLC–MS/MS analysis were labeled with numbers. (B) Western blotting analysis of synaptotagmin I and Rab3A. Left, Western blotting analysis of synaptotagmin I immunoprecipitated with Rab3A-specific antibody from rat brain synaptosome extract; Right, Western blotting analysis of Rab3A immunoprecipitated with synaptotagmin I-specific antibody from rat brain synaptosome extract.

3.3. Effect of calcium ion on Rab3A interaction with synaptotagmin I

To determine the effect of Ca^{2+} on Rab3A interaction with synaptotagmin I, rat brain synaptosomes were separately extracted in the presence of 2 mM EGTA or 150 μM $CaCl_2$ for immunoprecipitation. The result of Western blotting showed that Rab3A was efficiently co-immunoprecipitated with the antibody specific for synaptotagmin I under both EGTA and Ca^{2+} conditions (Fig. 2A), indicating that the interaction between the two proteins was not Ca^{2+}-sensitive. In addition, the effect of Ca^{2+} on the interaction between Rab3A and synaptototagmin I was also analyzed in pull-down method with the expressed GST-Syt I CR fusion protein (the cytoplasmic region of synaptototagmin I, residues 96–421). The results showed that Rab3A could be efficiently pulled down with GST-Syt I CR protein under both EGTA and Ca^{2+} conditions (Fig. 2B). These data demonstrated that the Rab3A interacts with synaptotagmin I in a Ca^{2+}-independent manner.

3.4. Synaptotagmin I domains involved in Rab3A binding

The primary sequence of synaptotagmin I reveals a large cytoplasmic region consisting of tandem C2 domains, C2A and C2B, which have been found to act as binding sites for Ca^{2+}, phospholipids and several effect proteins [17,18]. To determine whether C2 domains were involved in the binding to Rab3A, C2A and C2B domains were expressed as GST-fused proteins, immobilized by binding to Glutathione-Sepharose beads and incubated with native synaptosome protein extracts. The results showed that Rab3A could bind to both C2A and C2B domains (Fig. 3A). Considering that the binding of the cytoplasmic part of synaptotagmin, C2AB, to the t-SNAREs was sensitive to ionic strength, the binding was specu-

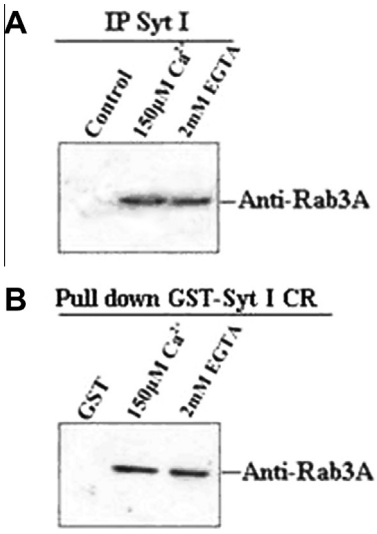

Fig. 2. Effect of calcium ions on the interaction of Rab3A with synaptotagmin I. (A) Rat brain synaptosome extract was immunoprecipitated with antibody against synaptotagmin I or non-immune serum in the presence of 150 μM $CaCl_2$ or 2 mM EGTA. (B) Rat brain synaptosome extract was pulled down with immobilized GST-Syt I CR (the cytoplasmic region of synatotagmin I) or GST alone in the presence of 150 μM $CaCl_2$ or 2 mM EGTA.

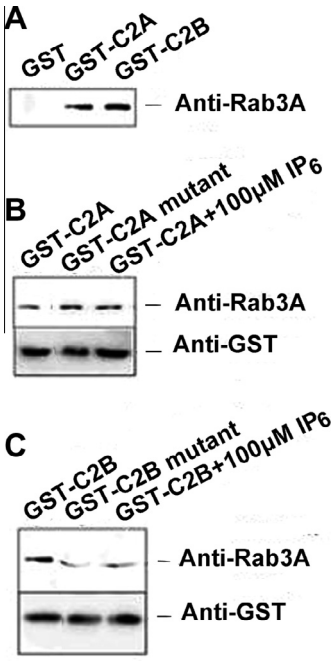

Fig. 3. Validation of synaptotagmin I domains and motifs involved in Rab3A binding. GST, Glutathione S-transferase; GST-C2A, GST-fused C2A domain; GST-C2A mutant, GST-fused C2A domain with mutated motif K189A/K190A/K191A/K192A; GST-C2B, GST-fused C2B domain; GST-C2B mutant, GST-fused C2B domain with mutated motif K324A/K325A/K3266A/K327A. IP_6, inositol 1,2,3,4,5,6-hexakisphosphate.

lated to be of electrostatic nature [19]. Syntaxin and SNAP-25, the main components of the SNARE complex, have been demonstrated to contribute acidic residues to a negatively charged surface on the SNARE complex [20], suggesting that the basic residues on synaptotagmin may be responsible for interaction with the t-SNARE complex. Therefore, the polylysine KKKK motifs (K189 to K192 in C2A and K234 to K237 in C2B) aroused our attention. Accordingly, we constructed and expressed the C2A and C2B domain mutants as GST fusion proteins, each with its KKKK motif mutated into AAAA. The results showed that the KKKK motif mutation in C2A domain did not affect the Rab3A binding to C2A domain, but the motif mutation in C2B domain obviously decreased the Rab3A binding to C2B domain (Fig. 3B and C), suggesting that the polybasic motif of C2B domain was a key site for Rab3A binding.

In order to further confirm this conclusion, we analyzed whether inositol 1,2,3,4,5,6-hexakisphosphate (IP_6) had an effect on the Rab3A interaction with C2 domains, considering that it had been well established that IP_6 could bind to the polylysine motif of C2B domain in synaptotagmin, which led to a decrease in exocytosis [21,22]. As a result, it was found that when C2A and C2B domain mutants were separately incubated with native synaptosome extract in the presence of 100 μM IP_6, C2A mutant exhibited normal Rab3A binding but the amount of Rab3A that was retained by C2B mutant was significantly decreased (Fig. 3B and C). These observations suggested that IP_6 competitively interfered with Rab3A interaction with C2B domain and the polybasic motif in C2B domain was indeed important for Rab3A binding whereas the polybasic motif in C2A domain was not involved in Rab3A binding.

3.5. Rab3A and syntaxin 1 competitively binding to C2B domain of synaptotagmin I

A SNARE is a SNAP (soluble NSF attachment protein) receptor and mediates the vesicle fusion during exocytosis. Exocytosis was found to necessitate the assembly of a tertiary complex between the vesicular SNARE (v-SNARE) and the target SNARE (t-SNARE) [4]. Syntaxin 1, as a very important component of t-SNARE complex, has been shown to be capable of forming a complex with the synaptotagmin I and this complex formation serves as an essential step in excitation–secretion coupling of the vesicles [23]. It has been reported that synaptotagmin I and II interact with syntaxin/SNAP-25 dimer and a stretch of basic amino acid residues in C2B domain of synaptotagmins is responsible for this Ca^{2+}-independent interaction, which is modulated by IP_6 [19]. Interestingly, the same polybasic motif in the C2B domain of synaptotagmin I was demonstrated in the present study to also mediate its interaction with Rab3A. In order to investigate if Rab3A and syntaxin 1 competitively occupy the same binding site in the C2B domain, we made competitively binding assays. At first, Glutathione-Sepharose beads-bound GST-C2B domain (5 μg) and a constant amount of purified recombinant His_6-tagged full length Rab3A (3 μg) were incubated with different concentrations of syntaxin 1B (0–1.25 μM final concentration). Western blotting analyses showed that, as the concentrations of syntaxin 1B increased, the signal intensity of GST-C2B-bound Rab3A decreased gradually (Fig. 4A). Conversely, when the amount of syntaxin 1B was fixed (16 μg), the amounts of syntaxin 1B that bound to C2B domain decreased as the increase of Rab3A concentration (Fig. 4B). These results demonstrated that the Rab3A and syntaxin 1B competed for binding to the same site in C2B domain of synaptotagmin I. In addition, in view of the fact that all the proteins used in the assays were purified in advance, it could be concluded that there were direct interactions between Rab3A as well as syntaxin 1B and the C2B domain of synaptotagmin I.

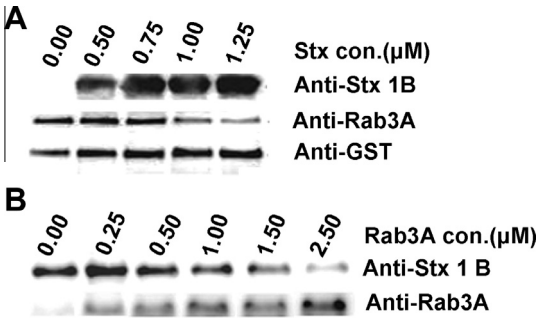

Fig. 4. Competitive binding of Rab3A and syntaxin 1B to the C2B domain of synaptotagmin I. GST-C2B (5 μg) bound to Glutathione-Sepharose beads was incubated with a constant amount of the purified recombinant His_6-tagged Rab3A and increasing amounts of His_6-tagged syntaxin 1B (A), or incubated with a constant amount of the purified recombinant His_6-tagged syntaxin 1B and increasing amounts of His_6-tagged Rab3A (B). After washing, the amounts of Rab3A and syntaxin 1B bound to C2B domain were detected.

4. Discussion

Many independent studies have demonstrated that synaptotagmin I, the major brain isoform, can interact specifically with the neuronal SNAREs in the absence of calcium [18]. As a Ca^{2+} and phospholipid binding protein, synaptotagmin I is essential for the synchronous synaptic vesicle exocytosis. The function of synaptotagmin I is regulated by its binding partners and several proteins have been found to bind to synaptotagmin I in a Ca^{2+}-independent manner, including clathrin, AP-2, neurexins, a family of neuronal cell surface proteins [24,25]. In the present study, proteomic analysis suggested that there are potential interaction between Rab3A and synaptotagmin I. Our study focused on the interaction between the two proteins mainly because (i) Rab3 and synaptotagmin have been reported to have opposite actions but cooperatively play critical regulatory roles in selecting and limiting the number

of vesicles released at central synapses, and (ii) Rab3A is one of the most abundant Rab3 isoforms in brain and has been found to be implicated in multiple steps of exocytosis by interaction with multiple factors through the same effector domain [26,27]. Rab3A protein belongs to the ras superfamily of small GTP-binding proteins. It can be either attached to membranes by a C-terminal lipid modification or be soluble in cells, suggesting that there may be a mechanism that removes the protein from membranes [10], thus neatly exerting its regulating function. To the best of our knowledge, this is the first comprehensive demonstration of the interaction between the two proteins.

Our results show that Rab3A is a new synaptotagmin I interacting partner and can directly bind to synaptotagmin I in a Ca^{2+}-independent manner, supported by the fact that the purified Rab3A and C2B domain of synaptotagmin I could bind to each other in the competition experiments. When we investigated the molecular mechanism underlying their interaction, it was found that polybasic motif KKKK in the C2B domain of synaptotagmin I was an important binding site, which was validated by mutation of polybasic motif and competition experiments with IP_6. It is worthy noting that, after the polybasic motif mutation, C2B domain still exhibited weak binding to Rab3A protein, suggesting that the polybasic motif KKKK was an important but not the whole site for the C2B binding to Rab3A and there were other non-electrostatic forces between C2B domain and Rab3A. This conclusion was supported by the failure to completely inhibit the binding with 100 μM IP_6. In contrast, the polybasic motif KKKK in C2A domain was shown to be not related to the domain binding to Rab3A, suggesting that the polylysine motifs in C2A and C2B domains had differences in the spatial distribution and thus the interaction with adjacent amino acid residues.

It was found in the present study that Rab3A, like IP_6, could Ca^{2+}-independently bind to the polybasic motif in C2 domain of synaptotagmin I and competitively affect the interaction between synaptotagmin I and syntaxin 1B that was involved in membrane fusion and vesicle exocytosis. The amount of Rab3A that bound to the C2B domain of synaptotagmin I was changed conversely to that of C2B domain-bound syntaxin and vise versa. The data suggested that Rab3A could participate in the regulation of the synaptic vesicle exocytosis by competitively modulating the interaction between synaptotagmin and SNARE complex in presynaptic membranes.

Competing interest

The authors declare that they have no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.090.

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