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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