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Interaction of NAP-22 with brain glutamic acid decarboxylase (GAD)

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HIGHLIGHTS

- GAD65 and GAD67 were immunoprecipitated using anti-NAP-22 antibody.
- NAP-22 binds GAD65 and GAD67 in vitro.
- NAP-22 showed no effect on the GAD activity.
- Co-localization of these proteins at the presynaptic region was observed.

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ABSTRACT

NAP-22 (also called BASP1 or CAP-23) is a neuron-enriched protein localized mainly in the synaptic vesicles and the synaptic plasma membrane. Biochemically, it is recovered in the lipid raft fraction. In order to understand the physiological function of the neuronal lipid raft, NAP-22 binding proteins were screened with a pull-down assay. Glutamic acid decarboxylase (GAD) was detected through LC–MS/MS, and Western blotting using a specific antibody confirmed the result. Two isoforms of GAD, GAD65 and GAD67, were expressed in bacteria as GST-fusion forms and the interaction with NAP-22 was confirmed in vitro. Partial co-localization of NAP-22 with GAD65 and GAD67 was also observed in cultured neurons. The binding showed no effect on the enzymatic activity of GAD65 and GAD67. These results hence suggest that NAP-22 could participate in the transport of GAD65 and GAD67 to the presynaptic termini and their retention on the synaptic vesicles as an anchoring protein.

1. Introduction

NAP-22 (CAP-23 and BASP1) is a neuron-enriched membrane protein localized in the presynaptic membrane regions such as the synaptic plasma membrane and the synaptic vesicles [9,13–15,18]. This protein is also a major component of the detergent-resistant membrane microdomain (DRM). Since cholesterol extraction with methyl-β-cyclodextrin resulted in the specific solubilizaiton of NAP-22 from DRM, cholesterol distribution and the localization of this protein was considered intimately related. Further studies elucidated the cholesterol binding activity of NAP-22 [16,24]. Recently, another functions of this protein in the process of transcriptional regulation, apoptosis, and ion channel formation were reported [7,8,20,21].

In order to elucidate additional functions of NAP-22, binding-protein screening assays using NAP-22-coupled Sepharose beads and immunoprecipitation using anti-NAP-22 antibody, coupled with LC–MS/MS, were performed. Two proteins, CapZ, an actin-binding protein, and synaptojanin-1, presynaptic phosphatydilinositol 4,5-bisphoshate phosphatase, were identified so far. The interactions of these proteins with NAP-22 were confirmed through further studies [19,23].

During these immunoprecipitation and pull-down assays, binding of glutamic acid decarboxylase (GAD) was also detected and Western blotting using anti-GAD antibody confirmed the result. The inhibitory neurotransmitter, γ-amino butyric acid (GABA), is synthesized by two isoforms of the enzyme glutamic acid decarboxylase (GAD): GAD65 and GAD67. These two proteins were expressed as GST-fusion proteins and the interaction was confirmed in vitro.

Abbreviations: TME buffer, 10 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM EGTA, pH 7.5; IP, immunoprecipitation; PMSF, phenylmethylsulfonyl fluoride; SPM, synaptic plasma membrane fraction; SVs, synaptic vesicles.

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Since considerable amount of NAP-22 localizes on the synaptic vesicle [28], the binding to NAP-22 could be a mechanism of the recruitment of GAD on the synaptic vesicle.

2. Materials and methods

All experimental protocols were performed in accordance with the guidelines for animal research of the Physiological Society of Japan and this study was approved by the Institutional Animal Care and Use Committee (permission number; 23-10-01) and carried out according to the Kobe University Animal Experimentation Regulations. All biochemical procedures were performed at 0–4 °C unless otherwise described.

2.1. Preparation of brain extracts and brain protein purification

For the pull-down assay of the crude extract, whole brains were homogenized in 6 vol. of 10 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM EGTA, pH 7.5 (TME buffer) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 100,000 × *g* for 1 h. The supernatant was then fractionated with 60% ammonium sulfate precipatation. The pellet fraction was dialyzed to TME buffer and used for pull-down assay after centrifugation at 20,000 × *g* for 40 min. Synaptic plasma membrane (SPM) preparation from the synaptosomal fraction of 6-week-old rat (Wistar) brains was done as described previously [19]. In brief, rat brains were homogenized in 8 vol. (v/w) of 0.32 M sucrose, 5 mM Hepes-KOH, 1 mM EGTA, 1 mM PMSF, and pH 7.5. After centrifugation at 3000 × *g* for 10 min, the supernatant was collected and centrifuged at 20,000 × *g* for 20 min. The pellet fraction (synaptosome fraction) was homogenized in 10 vol. TME buffer containing 1 mM PMSF. After stirring for 30 min, the homogenate was centrifuged at 25,000 × *g* for 40 min, and the pellet fraction (SPM; synaptosomal plasma membrane fraction) was recovered. The supernatant fraction was further centrifuged at 200,000 × *g* for 2 h, and the pellet was recovered as the synaptic vesicle fraction. The SPM and the synaptic vesicle fraction were homogenized in TME buffer containing 2% Triton X-100 and centrifuged at 100,000 × *g* for 60 min. The supernatants were used for immunoprecipitation assay (IP assay). The condition of pull-down and IP assay was described previously [14,19]. NAP-22 was prepared from rat brain as described [16].

2.2. Expression, purification, and pull-down analysis of artificially expressed proteins

Human GAD isoforms were expressed as the GST fusion proteins and purified using GSH-Sepharose beads as described [6]. Purified proteins were dialyzed to the GAD assay buffer (100 M phosphate buffer, pH 7.2, 15 μM pyridoxal phosphate) and stored on ice until use.

2.3. Assay of GAD activity

GST-GAD65 and GST-GAD67 were used to study the effect of NAP-22 on the GAD activity. The activity was assayed in the GAD assay buffer containing 4 mM glutamate and 40 nM δ-aminovaleric acid (internal standard) at 37 °C for 60 min. The production of GABA was assayed using a HPLC system (BioCAD700E, GE Healthcare) equipped with a Nucleosil 100 C18 AB column (250 mm × 4 mm, Kanto Kagaku Co., Japan) after a precolumn derivation of GABA which reacted with *o*-phthaldialdehyde (OPA) reagent to form a fluorescent compound [3,4]. Less than 5% of the glutamate was converted to GABA and the reaction was time-, and dose-dependent under the assay condition. The purity of the GAD in the fractions was calculated through densitometric scans of the SDS-PAGE gels.

2.4. Immunostaining of neurons

Primary culture of neonatal rat cortex neuron was performed as described [25]. Cells (12 day in culture) were fixed with 3.7% formaldehyde/PBS for 10 min at 37 °C. After washing twice with PBS containing 10 mM glycine, cells were incubated in 0.1% Triton X-100 in PBS for 10 min, and then washed twice. Blocking was done with 1% goat and/or rabbit serum in PBS for over 30 min at RT. Primary antibodies were then applied and incubated for 30 min at RT. The cover glass was then washed three times (each 10 min) with PBS and second antibodies (Alexa 488 or 546 coupled) were applied and incubated for 30 min at RT. After three times wash with PBS, samples were sealed with Vectashield (Vector Lab. CA). Olympus FV-1000 was used for observation.

2.5. Others

Protein concentration measurement, SDS-PAGE, and Western blotting were done as described previously [14,17,22]. LC–MS/MS analysis of the protein band was done as described [29]. An anti-GAD antibody reactive to GAD65/67 was purchased from Sigma (G5163). For immunostaining and Western blotting, anti-GAD65 (GAD2; cell signaling Tech.) and anti-GAD67 (GenScript) were also used. Preparation of anti-NAP-22 antibodies was described previously [15].

3. Results

In previous studies, pull-down assays using NAP-22-coupled Sepharose beads were performed and CapZ and synaptjanin-1 were identified as binding partners of NAP-22. In this study, in order to screen NAP-22 binding proteins in the crude soluble fraction, we attempted another pull-down assay using an anti-NAP-22 monoclonal antibody. Since almost all part of NAP-22 is localized in the membrane fraction in adult brain, the recovery of very small amount of NAP-22 in the pull-down sample from the crude soluble fraction was anticipated [16]. Half part of the antibody beads was hence supplied with enough amount of purified NAP-22 in advance. SDS-PAGE analysis of the precipitates showed that the amount of endogenous NAP-22 recovered in the pellet is very small compared to the NAP-22 supplied beads as anticipated (Fig. 1A, lane 1 vs. lane 2, shown with an arrow). In addition to NAP-22, many proteins were detected in the fractions and some proteins were enriched in lane 2. These bands were analyzed with LC–MS/MS and one band was found to contain glutamate decarboxylase (GAD) of 67 kDa (GAD67) (Fig. 1A, lane 2, shown with an arrowhead). The presence and enrichment of GAD was confirmed with Western blotting. In contrast to a very faint band in lane 1, two proximate bands were recognized in lane 2 (Fig. 1B). Since this antibody is reported to recognize both GAD65 and GAD67, the two bands were assumed to correspond to these proteins. Binding of both isoforms of GAD to synaptic vesicles (SVs) is well recognized and the localization of NAP-22 on the SVs is also shown [9,28]. Additional IP assay was then performed using Triton-solubilized SV fraction. In this case, anti-NAP-22 antibody beads were used without additional NAP-22 and Sepharose 4B beads were used as a control (Fig. 1C). Many proteins including NAP-22 were recovered in the IP fraction and the enrichment of some protein bands was also observed (Fig. 1C, lane 2). In this case, the band corresponding to GAD was evident and the enrichment of GAD65 and GAD67 was confirmed using specific antibodies to these proteins (Fig. 1D).

In order to confirm direct interaction of NAP-22 to these proteins, GST-GAD65 and GST-GAD67 were expressed in bacteria and purified. Binding of these proteins to NAP-22-charged