Neuroscience Letters 675 (2018) 59–63

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Interaction of dynamin I with NAP-22, a neuronal protein enriched in the presynaptic region

Satoko Ueno[a], Hiroshi Miyoshi[b], Yoko Maruyama[a], Mitsuhiro Morita[a], Shohei Maekawa[a,*]

[a] Department of Biology, Graduate School of Science, Kobe-University, Kobe, 657-8501, Japan
[b] Department of Microbiology, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae, Kawasaki, 216-8511, Japan

ARTICLE INFO

Keywords:
Dynamin
NAP-22
Calmodulin
Phosphatidylserine
Endocytosis

ABSTRACT

Neurons have well-developed membrane microdomains called “rafts” that are recovered as a detergent-resistant low-density membrane microdomain fraction (DRM). NAP-22 is one of the major protein components of neuronal DRM and localizes in the presynaptic region. In order to know the role of NAP-22 in the synaptic transmission, NAP-22 binding proteins in the cytosol were searched with an affinity screening with NAP-22 as a bait and several protein bands were detected. Using mass-analysis and western blotting, one of the main band of ~90 kDa was identified as dynamin I. The GTPase activity of dynamin I was partly inhibited by NAP-22 expressed in bacteria and this inhibition was recovered by the addition of calmodulin, a NAP-22 binding protein. The GTPase activity of dynamin was known to be activated with acidic membrane lipids such as phosphatidylserine and the addition of NAP-22, a phosphatidylserine binding protein, inhibited the activation of the GTPase by this lipid. Since NAP-22 localizes on the presynaptic plasma membrane and on synaptic vesicles, these results suggest the participation of NAP-22 in the membrane cycling through binding to dynamin and acidic membrane lipids at the presynaptic region.

1. Introduction

The exocytosis of neurotransmitters and the endocytosis of the membrane of the synaptic vesicles are performed at the presynaptic region of the nervous system. Proteins that participate in these membrane dynamics are hence considered to be enriched in this region. In fact, various proteins in the presynaptic region were found to have specific roles in this process [21,2].

Detergent-resistant membrane microdomain (DRM) is a membrane subdomain recovered in a low-density fraction after non-ionic detergent (such as Triton X-100) extraction of cell membrane and density gradient centrifugation at low temperature (0–4 °C). Neuronal cell membrane is rich in DRM. NAP-22 (also called CAP23 or BASP1) is a neuron-enriched calmodulin (CaM) binding protein localized in the presynaptic region and one of the major proteins of rat brain DRM [12–14,27,7]. In order to elucidate the role of NAP-22 in neurons, its interacting partners were screened using the immunoprecipitation method and several proteins such as synaptojanin1, CapZ, GAD, and calcineurin were identified in previous studies [23,18,11,8]. In this study, another interaction protein, dynamin I, was identified in the crude cytoplasmic extract of rat brain through the affinity screening with NAP-22 as a bait. Since NAP-22 inhibited the GTPase activity of brain-derived or bacteria-expressed dynamin, this protein may have some regulatory role in the endocytosis at the presynaptic region.

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; 26-10-02) 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 affinity screening (AS) of the crude brain extract, brains of Wistar rat (~4-week-old) were homogenized in 3 vol of TME solution (10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM EGTA, pH 7.4) containing 1 mM

Abbreviations: DRM, detergent-resistant low-density membrane microdomain fraction; CaM, calmodulin; TME, solution 10 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM EGTA, pH 7.4; PMSF, phenylmethylsulfonyl fluoride; AS, affinity screening; AS buffer, 10 mM Tris-HCl, 1 mM $MgCl_2$, 0.2 mM EGTA, 140 mM NaCl, pH 7.4; SAS, saturated ammonium sulfate; PS, phosphatidylserine; PIP_2, phosphatidylinositol 4,5-bisphosphate

* Corresponding author at: Department of Biology, Graduate School of Science, Kobe-University, Rokkodai-cho 1-1, Nada-ku, Kobe 657-8501, Japan.
E-mail address: smaekawa@kobe-u.ac.jp (S. Maekawa).

https://doi.org/10.1016/j.neulet.2018.03.061
Received 27 February 2018; Received in revised form 24 March 2018; Accepted 28 March 2018
Available online 28 March 2018
0304-3940/

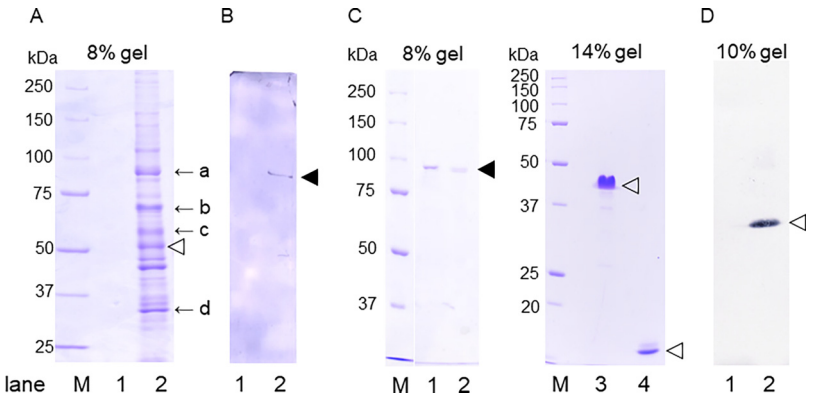

Fig. 1. AS with NAP-22 as a bait and preparation of proteins. A. SDS-PAGE pattern of the AS. 30% SAS precipitate fraction of the crude extract of rat brain was mixed with anti-NAP-22 antibody beads (lane 1) or anti-NAP-22 antibody beads premixed with NAP-22 (lane 2) and the precipitates were prepared. M; marker proteins (Bio-Rad). Four major bands (indicated by arrows) were further analyzed with LC–MS/MS. The most possible candidate of these bands was as follows. Band a; dynamin I, band b; V-type proton ATPase catalytic subunit A, band c; CaM-dependent protein kinase II, band d; heterogeneous nuclear ribonucleoproteins. The band of NAP-22 is indicated with an arrowhead. B. Western blotting pattern of "A" using anti-dynamin antibody (D25520). The band of dynamin is shown with an arrowhead. C. Purified dynamin fractions from rat brain (lane 1) and from expressed bacteria (lane 2). Purified NAP-22 (lane 3) and CaM (lane 4) from expressed bacteria, and molecular mass markers (lane M) are also shown. D. Binding of NAP-22 to dynamin-coupled Sepharose beads (2) or control beads (1). NAP-22 is shown with an arrowhead.

phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 100,000 xg for 1 h. The supernatant was then fractionated with 30% saturated ammonium sulfate (SAS) precipitation and the pellet fraction was dialyzed as described previously [8].

Dynamin I was purified from rat brain using affinity chromatography of amphiphysin II-SH3 domain as described [19,15]. Purification of NAP-22 from rat brain DRM was done as described [8,14].

2.2. Expression and purification of artificially expressed proteins

Bacterial expression and purification of mouse dynamin I was performed according to our previous reports [22,25]. Bacterial expression and purification of CaM and myristoylated NAP-22 was done as described [6,8].

2.3. AS with NAP-22 as a bait

For the AS with NAP-22 as a bait, CNBr-activated Sepharose 4 B was coupled with monoclonal anti-NAP-22 antibody (5-4E1). After mixing NAP-22 prepared from brain with the antibody beads for 2 h at 4 °C, unbound NAP-22 was removed by centrifugation. After washing the beads with the AS buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, 0.2 mM EGTA, 140 mM NaCl, pH 7.4) containing 0.2% Triton X-100, beads were mixed with 30%SAS fraction dialyzed to the AS buffer and mixed for 2 h at 4 °C. After washing four times with AS buffer containing 0.2% Triton X-100, beads were further washed twice with AS buffer. Beads were then mixed with 1vol of 2X-SDS-sample buffer without 2-mercaptoethanol at 56 °C for 20 min. After centrifugation at 3000xg for 2 min, the supernatant was recovered and boiled for 2 min in the presence of 2% 2-mercaptoethanol and used for SDS-PAGE.

2.4. Immunostaining of neurons

Immunostaining of neurons of 16 days in culture was done as described using newborn rat brain cortical neurons [8,15]. Anti-dynamin antibody (sc-6401) was obtained from Santa Cruz Biotech. Inc. For NAP-22 staining, anti-NAP-22 monoclonal antibodies (5-2D8) produced in our lab. were used [8,14,24]. Incubation with secondary antibodies Alexa-546 coupled anti-mouse antibody (Invitrogen) and Alexa-488 coupled anti-goat antibody (abcam) was done for 60 min at 37 °C. Samples were observed with a confocal microscope (Olympus FV-1000).

2.5. Statistical analysis

Data are presented as mean ± SD (n = 4). Student's *t*-test was used for statistical comparisons and are presented. $P < 0.05$ was considered statistically significant (*), $^{**}P < 0.01$.

2.6. Others

GTPase activity of dynamin was measured in a buffer (10 mM Tris-HCl, 10 mM NaCl, 2 mM $MgCl_2$, 0.05% Tween 80, pH 7.4) containing 0.3 mM GTP at 37 °C for 10 30 min [19]. In experiments studying the effect of CaM, 1.2 mM $CaCl_2$ and 1.0 mM EGTA were added to the reaction buffer (0.2 mM free $[Ca^{2+}]$). The reaction was stopped with the addition of EDTA (final 20 mM) and liberated Pi was assayed using the malachite green method [19,22]. Since both NAP-22 (prepared from brain and expressed in bacteria) showed basically same inhibitory effect on dynamin GTPase, bacteria-expressed NAP-22 was used throughout the inhibition assay. For immunoblotting of dynamin I, anti-dynamin antibody (D25520) (Millipore Inc.) was used. Biochemical analysis such as protein determination, SDS-PAGE, western blotting, was performed as described previously [14]. For LC–MS/MS analysis, protein bands of SDS-PAGE gels were excised and analyzed with Finnigan LTQ linear ion-trap mass spectrometers (ITMS; ThermoFischer, San Jose, CA, USA) after trypsin-digestion at the Research Facility Center for Science and Technology of Kobe-University. PS (porcine brain) was obtained from Avanti polar lipid Inc. (Canada).

3. Results

3.1. Identification of dynamin I in the AS with NAP-22 as a bait

In order to identify NAP-22 interaction proteins in the soluble fraction of rat brain, crude extract was fractionated with ammonium sulfate and the 30% SAS fraction was mixed with anti-NAP-22 antibody beads with or without prebinding of NAP-22. Since NAP-22 is a membrane bound protein, binding of solubilized NAP-22 to anti-NAP-22 antibody coupled to the Sepharose beads was considered to be necessary for the detection of its interacting proteins in the soluble fraction. Fig. 1A shows the SDS-PAGE pattern of the precipitates. In a control lane (lane 1), in which the antibody beads were not mixed with NAP-22, little protein bands were observed. In contrast, several protein bands were clearly detected in the precipitate, in addition to NAP-22 that was added to the antibody beads in advance (lane 2). LC–MS/MS analysis showed that the band "a" of ~90 kDa was found to contain dynamin I and western blotting using anti-dynamin antibody confirmed this result (Fig. 1B).

3.2. Interaction of NAP-22 and dynamin

Dynamin I is a 96 kDa protein having GTPase activity principally involved in the scission of newly formed vesicles from the plasma membrane. Dynamin I is recruited to the sites of clathrin-mediated endocytosis by amphiphysin and pinch off the membrane through polymerization and interaction to the membrane lipids [1,3]. In order to study the interaction *in vitro*, dynamin I was then purified from rat