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Identification of dynamin as a septin-binding protein

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HIGHLIGHTS

- Septin from lipid raft of rat brain contained several isoforms forming heterooligomers.
- Dynamin was shown to be co-fractionated with septin from lipid raft.
- Dynamin fraction prepared from rat brain contained several septin isoforms.
- Bacterially expressed septin5 and septin11 bound dynamin, but septin9 did not.
- Partial co-localization of septin5 and dynamin was observed in cultured neurons.

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ABSTRACT

Lipid rafts (detergent-resistant low-density membrane microdomain: DRM) are signal-transducing membrane platforms. In a previous study, we showed maturation-dependent localization of septin in the DRM fraction of rat brain. Mammalian septin is composed with 13–14 isoforms and these isoforms assemble to form rod-shaped hetero-oligomeric complexes. End-to-end polymerization of these complexes results in the formation of higher order structures such as filamentous sheets or bundles of filaments that restrict the fluid-like diffusion of the membrane proteins and lipids. Considering the function of septin as the membrane scaffold, elucidation of the molecular interaction of septin in DRM could be a breakthrough to understand another role of lipid rafts. In order to identify septin-binding proteins in DRM, solubilization and fractionation of septin from DRM was attempted. Several proteins were co-fractionated with septin and LC–MS/MS analysis identified one of these proteins as dynamin and Western blotting using anti-dynamin confirmed this result. Immunoprecipitation of septin11 in a crude supernatant showed co-precipitation of dynamin and dynamin fraction prepared from brain contained several septin isoforms. Within bacterially expressed septin isoforms, septin5 and septin11 bound dynamin but septin9 did not. These results suggest that some septin isoforms participate in the dynamin-related membrane dynamics.

1. Introduction

The presence of various membrane microdomains has been widely accepted through the works on the "lipid raft hypothesis" [7]. Some of the microdomains are thought to be recovered in the detergent-resistant low-density microdomain fraction (DRM), because membrane cholesterol and sphingolipids are highly enriched in this fraction [2]. Since many signal-transducing proteins are localized, DRM is considered as a small and transient platform of cell signaling. Identification of DRM-enriched proteins and elucidation of their interactions are hence important in order to understand the molecular background of the cell functions. In non-neuronal cells and tissues, very small amount of membrane protein (0.1–0.05%) is recovered in DRM. In contrast, nearly 10% protein of the synaptic plasma membrane is recovered in DRM. Identification and elucidation of molecular interactions in DRM is therefore of primary importance to understand the functions of the neuronal membrane [8,12].

Septin was found as the component of the submembranous filamentous structure localized at the neck of the budding yeast. Further studies showed its partial co-localization with

Abbreviations: TME buffer, 10 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM EGTA, pH 7.5; TMEG buffer, TME buffer containing 0.2 mM GTP; TMENG buffer, TMEG buffer containing 0.15 M NaCl; PMSF, phenylmethylsulfonyl fluoride; HME buffer, 10 mM Hepes-KOH, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, pH 7.7; DRM, detergent-resistant low-density membrane microdomain fraction; LC–MS/MS, liquid-chromatography tandem mass spectrometry.

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microtubules and microfilaments in addition to the membrane [3,5]. Recent studies have not only provided structural information about the different levels of septin organization but also elucidated their roles as scaffolding proteins for proteins and lipids and as diffusion barriers that participate in the subcellular compartmentation in many biological processes [14]. The molecular mechanisms of septin filament assembly and its regulation are however poorly understood.

In a previous study, we showed maturation-dependent localization of septin isoforms in DRM [10]. In this study, further purification of septin from DRM was attempted through polymerization–depolymerization cycles and several column procedures and identified dynamin as a septin-binding protein.

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. Purification of septin from brain DRM fraction

Septin was solubilized and partially purified from DRM using extraction with 60 mM $MgCl_2$ solution and polymerization–depolymerization procedure as described [10]. After two cycles of polymerization–depolymerization, proteins were solubilized with 60 mM $MgCl_2$ solution and applied to a hydrophobic column (butyl-Toyopearl (Toyo-Soda Co., Tokyo)) after the addition of 2 M NaCl. The column was washed with TME buffer (10 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM EGTA, pH 7.5) containing 2 M NaCl, proteins were then eluted with TME buffer containing 0.4 M NaCl solution. The 0.4 M NaCl eluate was then applied to a hydroxyapatite (HAP) (Bio-Rad) column, and the column was washed with 150 mM phosphate buffer (pH 8.0). Proteins were then eluted with a linear gradient of phosphate buffer (150–500 mM). After SDS-PAGE and Western blotting, septin-containing fractions were collected and dialyzed to TMEG buffer (TME buffer + 0.2 mM GTP).

2.2. Expression of septin in bacteria and purification

Construction of plasmids, expression and purification of GST-septin and heterotrimeric septin complex (septin7/5/9) were described previously [11].

2.3. Immunoprecipitation of septin and its binding proteins

Rat brain was homogenized in HME buffer containing 0.25 M NaCl and 1 mM PMSF. After centrifugation at 20,000 × *g* for 30 min, the supernatant was used for immunoprecipitation using anti-septin11 coupled beads in HME buffer containing 0.15 M NaCl [10].

2.4. Purification of dynamin from rat brain

Dynamin was prepared from rat brain according to [17]. Brains were homogenized with 2 vol. (w/v) of buffer 1 (20 mM Tris–HCl, 1 mM $CaCl_2$, 2 mM DTT, 1 mM PMSF, pH 7.5). After centrifugation at 20,000 × *g* for 30 min, the pellet was homogenized again in 2 vol. of buffer 1 and re-centrifuged. The pellet fraction was then homogenized in buffer 2 (20 mM Tris–HCl, 2 mM EDTA, 2 mM EGTA, 250 mM NaCl, 1 mM PMSF, pH 7.5) and centrifuged. The supernatant was recovered and the pellet was homogenized again and centrifuged. The supernatants were combined and recovered as the crude dynamin fraction. This sample was then fractionated with 35% ammonium sulfate and the pellet fraction was dialyzed to buffer 3 (20 mM Tris–HCl, 1 mM EDTA, 200 mM NaCl, 1 mM DTT, 0.1% Tween 80, pH 7.5). After centrifugation, the supernatant was applied to a column of GSH beads containing GST-amphiphysinII-SH3. The column was washed with buffer 3 and buffer 3 without Tween 80. Dynamin was then eluted from the column with buffer 4 (20 mM Hepes-KOH, 1.2 M NaCl, 1 mM DTT, pH 6.5), dialyzed to buffer 5 (10 mM Hepes-KOH, 50 mM NaCl, 1 mM $MgCl_2$, 0.2 mM EGTA, 1 mM DTT, pH 7.6) and used immediately or stored at −80 °C after the addition of glycerol to 20%. The amount of dynamin II and III in this fraction is known to be less than 1% of dynamin I [17].

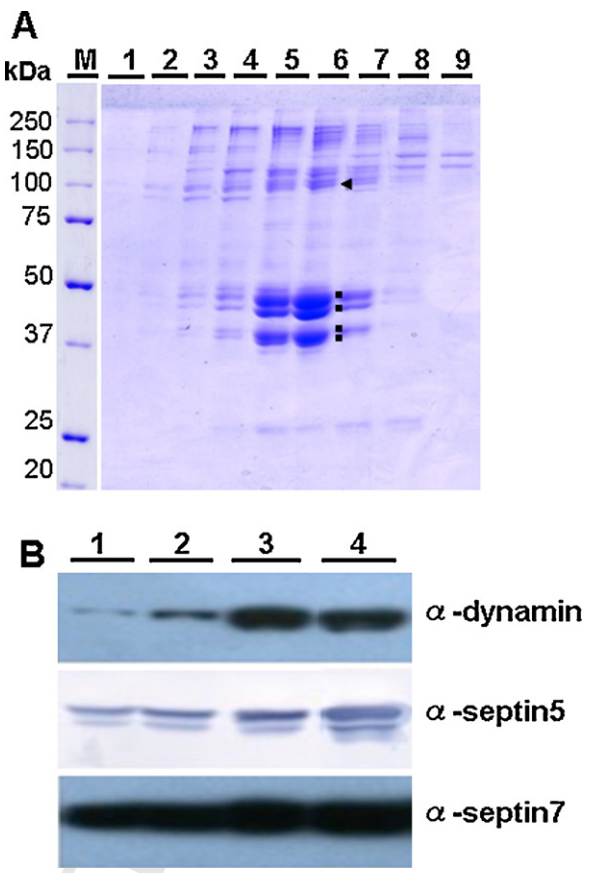

Fig. 1. Identification of dynamin in the septin fraction. (A) An elution pattern of septin through HAP column under the gradient of 240–450 mM of phosphate buffer. The SDS-PAGE pattern of the eluates (fraction # 1–9) and molecular mass markers (lane M) (Bio-Rad) is shown. A band shown with an arrowhead contained dynamin and septin isoforms are shown with square dots. (B) Western blotting analysis of dynamin through septin preparation. Equal protein amount of DRM fraction (lane 1), one-cycled polymerization–depolymerization fraction (lane 2), two-cycled fraction (lane 3), and septin fraction after HAP column (lane 4) were analyzed. Dynamin and septin7 were detected using ECL system and septin5 was detected using alkaline phosphatase system (Promega).

2.5. Immunostaining of neurons

Primary culture of neonatal rat cortex neuron was performed as described [22]. After 14 days in culture, cells were immunostained after fixation with 3.7% formaldehyde/PBS for 10 min at 37 °C.

2.6. Others

SDS-PAGE, Western blotting, and protein determination were done as described previously [9,10]. Antibodies to septin3

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