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Docosahexaenoic acid-containing phosphatidic acid interacts with clathrin coat assembly protein AP180 and regulates its interaction with clathrin

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**Article info**

Article history:
Received 25 October 2021
Received in revised form 12 November 2021
Accepted 27 November 2021
Available online 29 November 2021

**Keywords:**
Phosphatidic acid
Phosphatidylinositol 4,5-bisphosphate
Clathrin-mediated endocytosis
Docosahexaenoic acid
AP180
Diacylglycerol kinase

**Abstract**

The clathrin coat assembly protein AP180 drives endocytosis, which is crucial for numerous physiological events, such as the internalization and recycling of receptors, uptake of neurotransmitters and entry of viruses, including SARS-CoV-2, by interacting with clathrin. Moreover, dysfunction of AP180 underlies the pathogenesis of Alzheimer’s disease. Therefore, it is important to understand the mechanisms of assembly and, especially, disassembly of AP180/clathrin-containing cages. Here, we identified AP180 as a novel phosphatidic acid (PA)-binding protein from the mouse brain. Intriguingly, liposome binding assays using various phospholipids and PA species revealed that AP180 most strongly bound to 1-stearoyl-2-docosahexaenoyl-PA (18:0/22:6-PA) to a comparable extent as phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), which is known to associate with AP180. An AP180 N-terminal homology domain (1–289 aa) interacted with 18:0/22:6-PA, and a lysine-rich motif (K38–K39–K40) was essential for binding. The 18:0/22:6-PA in liposomes in 100 nm diameter showed strong AP180-binding activity at neutral pH. Notably, 18:0/22:6-PA significantly attenuated the interaction of AP180 with clathrin. However, PI(4,5)P2 did not show such an effect. Taken together, these results indicate the novel mechanism by which 18:0/22:6-PA selectively regulates the disassembly of AP180/clathrin-containing cages.

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1. Introduction

Clathrin coat assembly protein AP180 is one of the accessory proteins mainly expressed in the brain [1]. AP180 is recruited to the plasma membrane by binding to phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) via its AP180 N-terminal homology domain (ANTH) [2]. Moreover, the C-terminal intrinsically disordered region (CID) of AP180 contains several clathrin-binding motifs, which function to accumulate clathrin at specific sites in the plasma membrane [3]. Thus, AP180 forms cages containing clathrin and is crucial for clathrin-mediated endocytosis (CME).

The CME has critical functions in all eukaryotic cells and regulates numerous important physiological events, such as receptor internalization, neurotransmitter uptake, signal transduction and pathogen elimination [4]. Moreover, CME is essential for the entry of viruses, including SARS-CoV-2 and influenza virus [5]. Furthermore, CME has been reported to be associated with the development of Alzheimer’s disease (AD) [6]. In this case, CME is closely involved in the internalization mechanism of extracellular amyloid precursor protein, which is toxic to nerve cells [7]. Because CME is physiologically and pathologically important as described above, it is important to understand the mechanisms of assembly and disassembly.
disassembly of the AP180/clathrin-containing cage. In particular, the disassembly mechanism of the cage is still elusive, although disassembly is essential for CME processes because release of endocytic machinery proteins is needed to initiate another endocytic event, and an uncoated vesicle can fuse with an endosome to prompt an intracellular trafficking event [8].

We recently found that the δ isozyme of diacylglycerol kinase (DGK), which transforms diacylglycerol to phosphatidic acid (PA) and consists of 10 isoforms [9,10], selectively produced docosahexaenoic acid-containing PA species (18:0/22:6-PA) in the brain [11]. However, the physiological functions and target proteins of 18:0/22:6-PA molecular species in the brain and nerve cells are largely unknown [12]. Therefore, we searched for the target protein(s) of 18:0/22:6-PA in the mouse brain.

In the present study, we identified AP180 as an 18:0/22:6-PA-binding protein. Interestingly, binding assays using various phospholipids-PA species-containing liposomes revealed that AP180 most intensely bound to 18:0/22:6-PA to a comparable extent as PI(4,5)P$_2$. Notably, 18:0/22:6-PA, but not PI(4,5)P$_2$, inhibited the interaction of AP180 with clathrin. These results shed light on the novel mechanism of disassembly of AP180/clathrin-containing cages induced by 18:0/22:6-PA.

2. Materials and methods

2.1. Materials

Lipids: 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (16:0/18:1-PC), 1,2-dipalmitoyl-sn-glycerol-3-phosphate (16:0/16:0-PA), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (16:0/18:1-PA), 1,2-dioleoyl-sn-glycero-3-phosphate (18:1/18:1-PA), 1-stearoyl-2-oleoyl-sn-glycero-3-phosphate (18:0/18:1-PA), 1,2-dioleoyl-sn-glycero-3-phosphate (18:0/18:0-PA), 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphate (18:0/20:4-PA), 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphate (18:0/22:6-PA), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (18:1/18:1-PE), 1,2-dioleoyl-sn-glycero-3-phosphoserine (18:1/18:1-PS), 1,3'-bis[1,2-dioleoyl-sn-glycero-3-phospho]-glycerol (18:1/18:1/18:1-CL), 1-stearoyl-2-archi donoyl-sn-glycero-3-phosphatidylinositol (18:0/20:4-PI), 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphoglycerol (18:0/22:6-PC) and 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphatidylinositol 4,5-bisphosphate (18:0/20:4-PI(4,5)P$_2$) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (Chol) was purchased from Wako Pure Chemical Industries (Osaka, Japan). 1,2-Dipalmitoyl-sn-glycero-3-phosphatidylinositol 4,5-bisphosphate (16:0/16:0-PI(4,5)P$_2$) was purchased from Echelon Biosciences (Salt Lake City, UT, USA).

Antibodies: Mouse monoclonal anti-His antibody (D291-3S) and rabbit polyclonal anti-GST antibody (PM013) were obtained from Medical and Biological Laboratories (Nagoya, Japan). Mouse monoclonal anti-clathrin-heavy chain (CHC) antibody (610499) was purchased from BD Transduction Laboratories (Lexington, KY).

2.2. Expression of recombinant proteins

Mouse AP180 cDNA was amplified using the primers 5'-GGTGGTCATATGCTGGGCACACCTG-3' (forward) and 5'-GGTCTCGAGTTACAAGAAATCCTTGATGTTAAG-3' (reverse) from mouse brain cDNA, ligated with pET28a or pGEX-6P-1 vector and then transfected into Rosetta 2 (DE3) Escherichia coli cells (Novagen, Merck, Darmstadt, Germany). The expression and purification of the 6 × His tag- and GST tag-fused proteins were performed as previously described [13,14].

2.3. Western blotting

Western blotting was performed as described previously [15].

2.4. Preparation of liposomes

Liposome preparation by sonication was performed as described previously [16]. Liposomes with different diameters of 100 nm, 400 nm or 1000 nm were produced by a Mini Extruder (Avanti Polar Lipids) [17].

2.5. Liposome binding assay

A liposome binding assay was carried out as described previously [16–18]. Purified 6 × His-tagged proteins (0.2 μM) were dissolved in HEPES buffer (25 mM HEPES, 100 mM NaCl, 1 mM DTT, pH 7.4) and incubated with liposomes at 4 °C for 30 min. After incubation, samples were ultracentrifuged at 200,000 g at 4 °C for 1 h. The precipitant was dissolved in HEPES buffer.

2.6. Neuro-2a cell culture

Neuro-2a mouse neuroblastoma cells were cultured as described previously [19].

2.7. Glutathione S-transferase (GST) pull-down assay

GST pull-down assays were performed as described previously [20].

2.8. Statistical analysis

Data are represented as the means ± S.D. and were analyzed using one-way ANOVA followed by Tukey’s post hoc test using

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**Figure 1.** Identification of AP180 as a PA-binding protein. (A) SCORE and emPAI of AP180. (B) Schematic diagram of wild-type (WT) AP180. (C) The 6 × His-AP180 protein expressed in E. coli cells was purified, separated by SDS–PAGE (6% acrylamide), and then stained with CBB or detected by WB with anti-His tag antibody. Liposome-binding assay of 6 × His-AP180 using D and E [16]: 10/60/30 mol% (X:Y:Z) and precipitate (P) were quantified by densitometry using ImageJ software (E and G). Binding activity was calculated as the percentage of the precipitate band intensity compared to the total band intensity. Values are presented as the mean ± SD of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.005 (versus 18:0/22:6-PA), one-way ANOVA followed by Tukey’s post hoc test.
Prism 8 (GraphPad Software) to determine any significant differences. p < 0.05 was considered significant.

3. Results

3.1. AP180 selectively binds to 18:0/22:6-PA and Pl(4,5)P2 but not with other phospholipids

After 18:0/22:6-PA-containing liposome precipitation from the mouse brain [17], liquid chromatography-tandem mass spectrometry identified the clathrin coat assembly protein AP180. The SCORE (probability of identification) and exponentially modified protein abundance index (emPAI) were 513 and 0.63, respectively (Fig. 1A). AP180 consists of ANTH (289 amino acids), which binds to Pl(4,5)P2, and CID (612 amino acids), which interacts with clathrin and forms cages, and is crucial for CME [2,3] (Fig. 1B).

We cloned AP180 cDNA from the reverse transcripts of mouse brain mRNAs and ligated it with the pET-28a vector. A 6×His-tag-fused AP180 protein was expressed in E. coli cells and purified by Ni²⁺-affinity chromatography. SDS–PAGE followed by Coomassie Brilliant Blue (CBB) staining showed that 6×-His-AP180 (approximately 150 kDa) was highly purified (Fig. 1C). Moreover, Western blotting (WB) using an anti-6×His antibody confirmed that the 150 kDa band, which is larger than the calculated molecular mass (91 kDa) (Fig. 1A), was 6×-His-AP180 (Fig. 1C).

We performed a liposome binding assay to investigate the binding activities of AP180 for several phospholipids, PC, PE, PS, CL, PI, PC, Pl(4,5)P2 or PA. As shown in Fig. 1D and E, more than 30% of 6×-His-AP180 was detected in the precipitate fractions of 18:0/22:6-PA, 16:0/16:0-Pl(4,5)P2 and 18:0/20:4-Pl(4,5)P2-containing liposomes. In addition, AP180 was mainly present in the supernatant at pH 6.2–7.4 when PC-, PE-, PS-, PI-, CL- and PG-containing liposomes were used. Approximately 70%, 30% and 80% of AP180 interacted with 18:0/22:6-PA, 16:0/16:0-Pl(4,5)P2 and 18:0/20:4-Pl(4,5)P2-containing liposomes, respectively (Fig. 1E).

Because the composition of fatty acyl moieties in PA molecular species possibly affects the binding activity of AP180, a liposome binding assay was conducted using liposome-containing 16:0/16:0-PA, 18:0/18:0-PA, 18:0/18:1-PA, 18:0/18:0-PA or 18:0/22:6-PA. As shown in Fig. 1F and G, all of the PA molecular species tested here showed at least 15% binding activities for AP180. Among them, 18:0/18:0-PA interacted with AP180 to a comparable extent as 18:0/22:6-PA (Fig. 1F and G).

3.2. 18:0/18:0-PA, 18:0/22:6-PA and Pl(4,5)P2 bind to the lysine-rich motif in ANTH of AP180

To determine the PA-interaction region of AP180, we executed a liposome binding assay using two deletion mutants of AP180 (6×-His-ANTH (1–289 amino acids) and 6×-His-CID (290–901 amino acids) that were expressed in E. coli cells and highly purified (Fig. 2A and B). As shown in Fig. 2C and D, 18:0/18:0-PA, 18:0/22:6-PA and Pl(4,5)P2 showed strong binding activities to 6×-His-ANTH, and their activities were comparable. However, 6×-His-CID did not show such interactions with 18:0/18:0-PA, 18:0/22:6-PA or Pl(4,5)P2 (Fig. 2E and F).

Previous studies indicated that ANTH directly interacts with Pl(4,5)P2 via its lysine-rich motif (K38–K93–K40) [2]. 6×-His-AP180-KE (K38–K93–K40 was mutated to E38–E93–E40) exhibited markedly reduced interactions with 18:0/18:0-PA, 18:0/22:6-PA and Pl(4,5)P2 (Fig. 2G and H). Therefore, these results indicate that the lysine-rich motif in ANTH is essential for the interaction with not only Pl(4,5)P2 but also PA and that Pl(4,5)P2 and PA bind to AP180 via the same site.

3.3. Characterization of the interaction between AP180 and PA

The physicochemical properties of anionic phospholipids such as PA and Pl(4,5)P2 are affected by pH [21,22]. As shown in Fig. 3A and B, the binding activity of AP180 to Pl(4,5)P2 gradually decreased with increasing pH. As previously reported [23], AP180, 18:0/18:0-PA and 18:0/22:6-PA strongly bound to AP180 in the range of pH 6.2–7.4 in which CME occurs.

The diameter of clathrin-coated vesicles is 60–120 nm [24]. We next performed liposome binding assays using liposomes with different diameters of 100 nm, 400 nm or 1000 nm. As shown in Fig. 3C and D, AP180 strongly bound to Pl(4,5)P2 independent of liposome diameter. The binding activities of 18:0/18:0-PA and 18:0/22:6-PA were moderately affected by liposome diameters.

The concentrations of Pl(4,5)P2 and PA in cell membranes are 0.2–1% and 1–5% of total lipids (mol%), respectively [25,26]. We next performed liposome binding assays using liposomes with different compositions of PA or Pl(4,5)P2. Approximately 95% and ~30% of AP180 interacted with 5 mol% Pl(4,5)P2 and 1 mol% Pl(4,5)P2 (Fig. 3G and H). Pl(4,5)P2-containing liposomes, respectively. Moreover, AP180 showed ~55% and ~10% binding activities to 5 mol% Pl(4,5)P2 and 1 mol% Pl(4,5)P2 (Fig. 3E and F). AP180 can bind to Pl(4,5)P2 and 18:0/22:6-PA under physiological conditions. Moreover, at lower concentrations, 18:0/22:6-PA more intensely interacted with AP180 than 18:0/18:0-PA (Fig. 3E–H), indicating that the affinity of AP180 for 18:0/22:6-PA is higher than that for 18:0/18:0-PA.

3.4. 18:0/22:6-PA attenuates the interaction of AP180 with clathrin heavy chain

To elucidate the physiological function of 18:0/22:6-PA for AP180 assembly with and disassembly from clathrin, pull-down experiments were performed. AP180-WT and AP180-KE were expressed in E. coli cells and highly purified as GST-fusion proteins (Fig. 4A). GST-AP180-WT and GST-AP180-KE were preincubated
with PL(4,5)P2 or 18:0/22:6-PA-containing liposomes and then incubated with Neuro-2a cell lysates, which contain CHC. CHC interacted with both GST-AP180-WT and GST-AP180-KD but not with GST alone (Fig. 4B and C). Notably, 18:0/22:6-PA significantly reduced the interaction of AP180 with clathrin (Fig. 4B and C). However, such decreases were not observed when AP180-KD, which lacks 18:0/22:6-PA- and PL(4,5)P2-binding activities (Fig. 2G and H), was used (Fig. 4B and C). Moreover, the reducing effect of PL(4,5)P2 on the AP180-clathrin interaction was not clearly detected (Fig. 4B and C). These results indicate that 18:0/22:6-PA selectively and effectively regulates the disassembly of clathrin from AP180.

4. Discussion

AP180, which is an assembly protein with clathrin, plays essential roles in CME [1–3]. In CME, not only the assembly of clathrin but also its disassembly is important [8]. However, compared to assembly machinery, little is known about the mechanism of clathrin cage disassembly [8]. In the present study, we have demonstrated for the first time that 18:0/22:6-PA selectively and strongly binds to AP180 to a comparable extent as PL(4,5)P2 (Figs. 1 and 3), which also interacts with AP180 and is essential for clathrin coat formation [1–3]. Moreover, our present data provide evidence indicating that 18:0/22:6-PA, but not PL(4,5)P2, functions as a selective and effective attenuator for the interaction of AP180 with clathrin (Fig. 4). Therefore, although 18:0/22:6-PA and PL(4,5)P2 bind to AP180 through the same site (Fig. 2), the lysine-rich motif (K38-K39-K40) in ANTH, the function of 18:0/22:6-PA is different from that of PL(4,5)P2.

The PKa values of D4- and D5-phosphate of PL(4,5)P2 and phosphate of PA are 6.5, 7.7 and 7.9, respectively [21,22]. Indeed, consistent with previous studies [23], the binding activity of PL(4,5)P2 to AP180 was decreased in a pH (6.2–7.4)-dependent manner (Fig. 3A and B). However, pH (6.2–7.4) did not substantially affect the AP180-binding activities of 18:0/18:0-PA and 18:0/22:6-PA. Therefore, 18:0/18:0-PA and 18:0/22:6-PA, but not PL(4,5)P2, maintain strong interactions with AP180 in the pH range in which CME occurs.

AP180 interacted with PL(4,5)P2-containing liposomes independent of their diameter (1000–10000 nm). However, the binding activities of AP180 with 18:0/18:0-PA and 18:0/22:6-PA were moderately altered depending on liposome diameter (Fig. 3C and D). In the case of 18:0/22:6-PA, AP180 showed stronger binding activities with larger liposomes. Therefore, the AP180-18:0/22:6-PA binding may prefer gentle membrane curvature of an uncoated vesicle fused with an early endosome (diameter: 400–1000 nm) rather than a clathrin-coated vesicle (diameter: ~100 nm).

The amount of PA (1–5%) in total cell lipids is approximately five times as large as that of PL(4,5)P2 (0.2–1%) [25,26]. AP180 showed ~55% binding activity to 5 mol% of 18:0/22:6-PA-containing liposomes and ~30% to 1 mol% of PL(4,5)P2-containing liposomes (Fig. 3E–H). Notably, the amount of 18:0/22:6-PA is significantly increased during neurite outgrowth [27]. Therefore, it is likely that 18:0/22:6-PA can more intensely associate with AP180 than PL(4,5)P2 and thus that the physiological effects of 18:0/22:6-PA on AP180 functions are comparable to or higher than those of PL(4,5)P2 in neuronal cells.

It was reported that inhibition of DGK attenuated CME [28]. Intriguingly, among DGK isozymes, DGKδ is related to CME according to a genome-wide associated study [29] and is distributed to clathrin-positive puncta [30] and early endosomes (Hoshino F. and Sakane F., unpublished work). Moreover, knockdown of DGKδ impaired CME in an enzymatic activity-dependent manner [30]. Notably, Lu et al. recently reported that DGKε selectively reduced 18:0/22:6-PA in the brain [11]. In the present study, 18:0/22:6-PA significantly attenuated clathrin assembly of AP180. We previously demonstrated that synaptojanin-1, which dephosphorylates PL(4,5)P2 to detach AP180 from the membrane and acts as a main player in clathrin disassembly [31], is activated by 18:0/22:6-PA [17]. Therefore, it is possible that 18:0/22:6-PA produced by DGKδ effectively regulates clathrin disassembly through dual targets, AP180 and synaptojanin-1. Inhibition of another PA-generating enzyme, phospholipase D (PLD), also reduces CME [32]. However, because PLD hydrolyzes PC, which contains primarily saturated and monounsaturated fatty acids, PLD likely does not contribute to 18:0/22:6-PA production.

In summary, in the present study, we identified AP180 as a novel 18:0/22:6-PA-binding protein. Moreover, we provided evidence that 18:0/22:6-PA, but not PL(4,5)P2, inhibits the interaction between AP180 and clathrin, strongly suggesting that this lipid species selectively and effectively enhances dissociation of clathrin from the AP180/clathrin complex cage. Our results shed light on a novel disassembly mechanism of AP180/clathrin-containing cages in CME, which underlies numerous physiological and pathological events, such as receptor internalization, neurotransmitter uptake, signal transduction, pathogen elimination, virus entry and AD.

Funding

This work was supported in part by grants from MEXT/JSPS, Japan (KAKENHI Grant Numbers: 17H03650 and 20H03205 [Grant-in-Aid for Scientific Research (B)] (F.S.), and 20J21133 [Grant-in-Aid for JSPS Fellows] (F.H.)); the Japan Food Chemical Research Foundation, Japan (F.S.); the SENSINH Medical Research Foundation, Japan (F.S.); the Uehara Memorial Foundation, Japan (F.S.); the Tojuro Iijima Foundation for Food Science and Technology, Japan (F.S.); and the Sugiyama Chemical and Industrial Laboratory, Japan (F.S.).
**Fig. 4.** 18:0/22:6:PA inhibits the interaction between clathrin and AP180. (A) The GST-AP180-WT and GST-AP180-KE (lys383/394/402lu) proteins expressed in E. coli cells were purified, separated by SDS–PAGE (6% acrylamide), stained with Coomassie Blue or detected by WB with anti-GST tag antibody. (B and C) Neuro-2a cell lysates were incubated with 10 μg of purified GST, GST-AP180-WT or GST-AP180-KE protein, and then 20 μl of gluthathione-Sepharose beads were added to buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, complete EDTA free protease inhibitor) with or without 100 nm liposomes (16:0/18:1-PC, 16:0/16:0-PE, 4:5:4:5:6-PC or 18:0/22:6:PA-liposomes (X/PC chols – 10/60/30 mol%) (X = PC, PA or PI(4,5)P2) (0.1 mM total lipid)). The beads were washed and recovered by low-speed centrifugation. (B) SDS–PAGE (10% acrylamide) was performed, and separated proteins were stained with Coomassie Blue and analyzed by WB using anti-GST antibodies. The amounts of (C) precipitates were quantified by densitometry using ImageJ software. Relative CHC levels were calculated as the percentage of the CHC band intensity precipitated with GST-AP180-WT or GST-AP180-KE with or without each liposome compared to the band intensity precipitated with GST-AP180-WT with no liposomes. Values are presented as the mean ± SD of three independent experiments. **p < 0.01, ***p < 0.005, one-way ANOVA followed by Tukey’s post hoc test.

**Authors’ contributions**

F.H. performed the experiments and analyzed the data. F.S. and F.H. wrote the manuscript. F.S. supervised the project. All authors reviewed and approved the final manuscript.

**Declaration of competing interest**

All authors have declared no conflicts of interest.

**Data availability**

Data will be made available on request.

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