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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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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 ($PI(4,5)P_2$), 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, $PI(4,5)P_2$ 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.

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 ($PI(4,5)P_2$) 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

Abbreviations: AD, Alzheimer's disease; ANTH, AP180 N-terminal homology domain; CBB, Coomassie Brilliant Blue; CID, C-terminal intrinsically disordered region; CHC, clathrin heavy chain; Chol, cholesterol; CL, cardiolipin; CME, clathrin-mediated endocytosis; DGK, diacylglycerol kinase; GST, glutathione S-transferase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PLD, phospholipase D; PS, phosphatidylserine; WB, Western blotting; WT, wild type.

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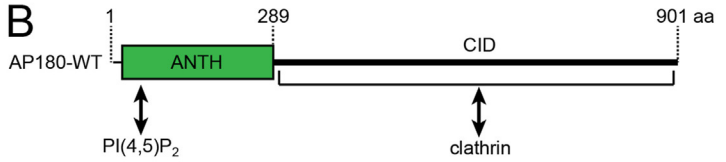
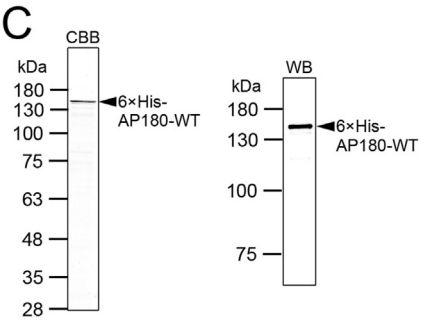
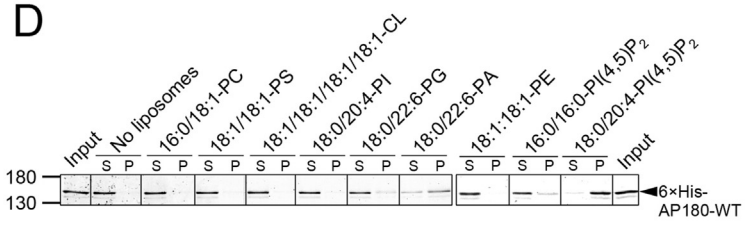
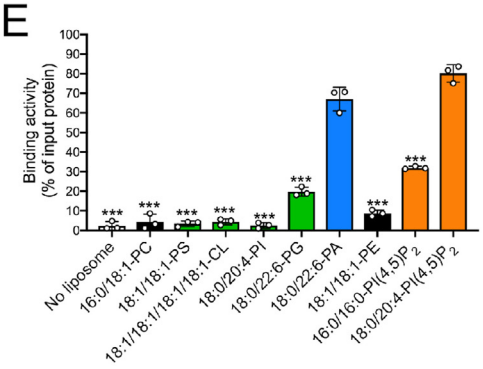
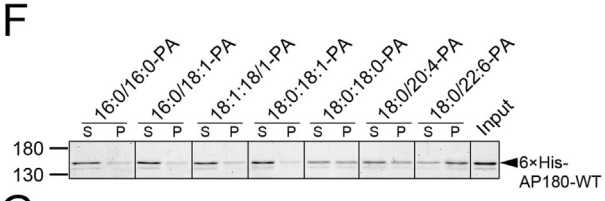
Protein name	M. M. (kDa)	SCORE	emPAI
Clathrin coat assembly protein AP180 (AP180)	91	513	0.63

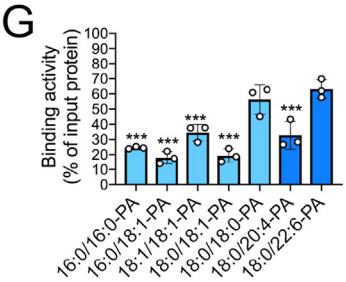












disassembly of the AP180/clathrin-containing cage. In particular, the disassembly mechanism of the cage is still elusive, although disassembly is essential for CME processes because release of endocytic machinery proteins is needed to initiate another endocytic event, and an uncoated vesicle can fuse with an early 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 isozymes [9,10], selectively produced docosahexaenoic acid-containing PA species (18:0/22:6-PA (X:Y = the total number of carbon atoms: the total number of double bonds in the fatty acyl moiety of the glycerol backbone)) 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- and 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-glycero-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-stealoyl-2-oleoyl-sn-glycero-3-phosphate (18:0/18:1-PA), 1,2-distearoyl-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 (18:1/18:1/18:1/18:1-CL), 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphatidylinositol (18:0/20:4-PI), 1-stearoyl-2-docosahexenoyl-sn-glycero-3-phosphoglycerol (18:0/22:6-PG) and 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphatidylinositol 4,5-bisphoaphate (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′-GGTGGTCATATGTCGGGCCAAACGCTC-3’ (forward) and 5′-GGTCTCGAGTTACAAGAAATCCTTGATGTTAAG-3’ (reverse) from mouse brain cDNA, ligated with pET-28a 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].

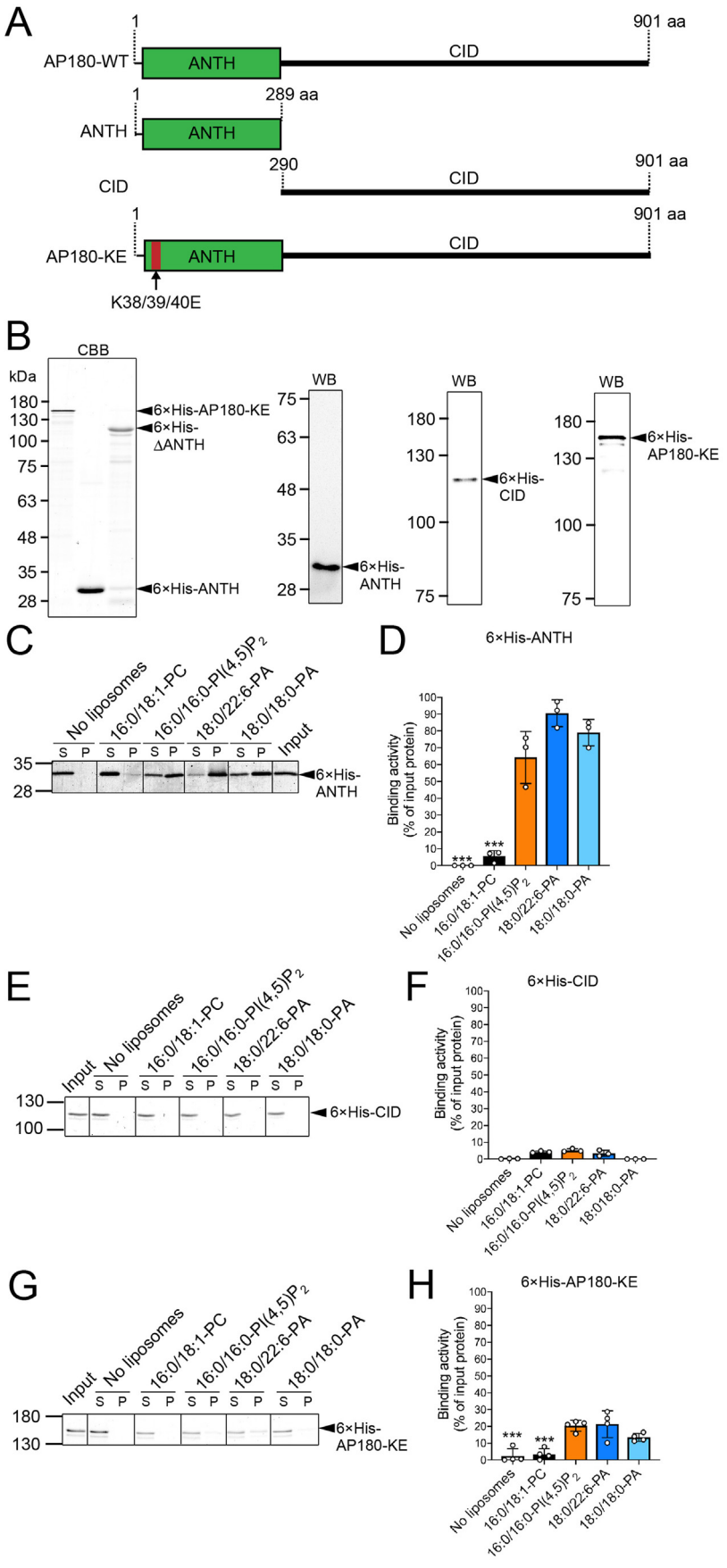
2.7. Glutathione S-transferase (GST) pull-down assay

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

Fig. 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) 16:0/18:1-PC, 18:1/18:1-PS, 18:1/18:1/18:1/18:1-CL, 18:0/20:4-PI, 18:0/22:6-PG, 18:0/22:6-PA, 18:1/17:1-PE, 16:0/16:0-PI(4,5)P_2 and 18:0/20:4-PI(4,5)P_2-liposomes (X/16:0/PC/chol = 10/60/30 mol% (X = PC, PS, CL, PI, PG, PE, PI(4,5)P_2)), and (F and G) 16:0/16:0-, 16:0/18:1-, 18:1/18:1-, 18:0/18:1-, 18:0/18:0-, 18:0/20:4- and 18:0/22:6-PA-liposomes (PA/PC/chol = 10/60/30 mol%). Purified 6×His-AP180 (0.2 μM) was incubated with each liposome (1 mM total lipids) and then separated by ultracentrifugation. SDS–PAGE (6% acrylamide) was performed, and separated proteins were stained with *CBB*. The positions of 6×His-AP180 are indicated with an arrowhead. The amounts of protein in the supernatant (*S*) 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.


A
AP180-WT
ANTH
CID
1
901 aa
289 aa
290
AP180-KE
K38/39/40E
B
CBB
kDa
6×His-AP180-KE
6×His-ΔANTH
6×His-ANTH
WB
6×His-CID
6×His-AP180-KE
C
No liposomes
16:0/18:1-PC
16:0/16:0-PI(4,5)P2
18:0/22:6-PA
18:0/18:0-PA
Input
S P
D
6×His-ANTH
Binding activity (% of input protein)
E
6×His-CID
F
G
6×His-AP180-KE
H

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 PI(4,5)P_2 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 PI(4,5)P_2, 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 Ni^{2+}-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, PG, PI(4,5)P_2 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-PI(4,5)P_2- and 18:0/20:4-PI(4,5)P_2-containing liposomes, while the protein was mainly present in the supernatant when PC-, PE-, PS-, PI-, CL- and PG-containing liposomes were employed. Approximately 70%, 30% and 80% of AP180 interacted with 18:0/22:6-PA-, 16:0/16:0-PI(4,5)P_2- and 18:0/20:4-PI(4,5)P_2-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, 16:0/18:1-PA, 18:1/18:1-PA, 18:0/18:1-PA, 18:0/18:0-PA or 18:0/20:4-PA, in addition to 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 PI(4,5)P_2 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 PI(4,5)P_2 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 PI(4.5)P_2 (Fig. 2E and F).

Previous studies indicated that ANTH directly interacts with PI(4,5)P_2 via its lysine-rich motif (K38–K39–K40) [2]. 6×His-AP180-KE (K38–K39–K40 was mutated to E38-E39-E40) exhibited markedly reduced interactions with 18:0/18:0-PA, 18:0/22:6-PA and PI(4,5)P_2 (Fig. 2G and H). Therefore, these results indicate that the lysine-rich motif in ANTH is essential for the interaction with not only PI(4,5)P_2 but also PA and that PI(4,5)P_2 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 PI(4,5)P_2 are affected by pH [21,22]. As shown in Fig. 3A and B, the binding activity of AP180 to PI(4,5)P_2 gradually decreased with increasing pH, as previously reported [23]. However, 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.

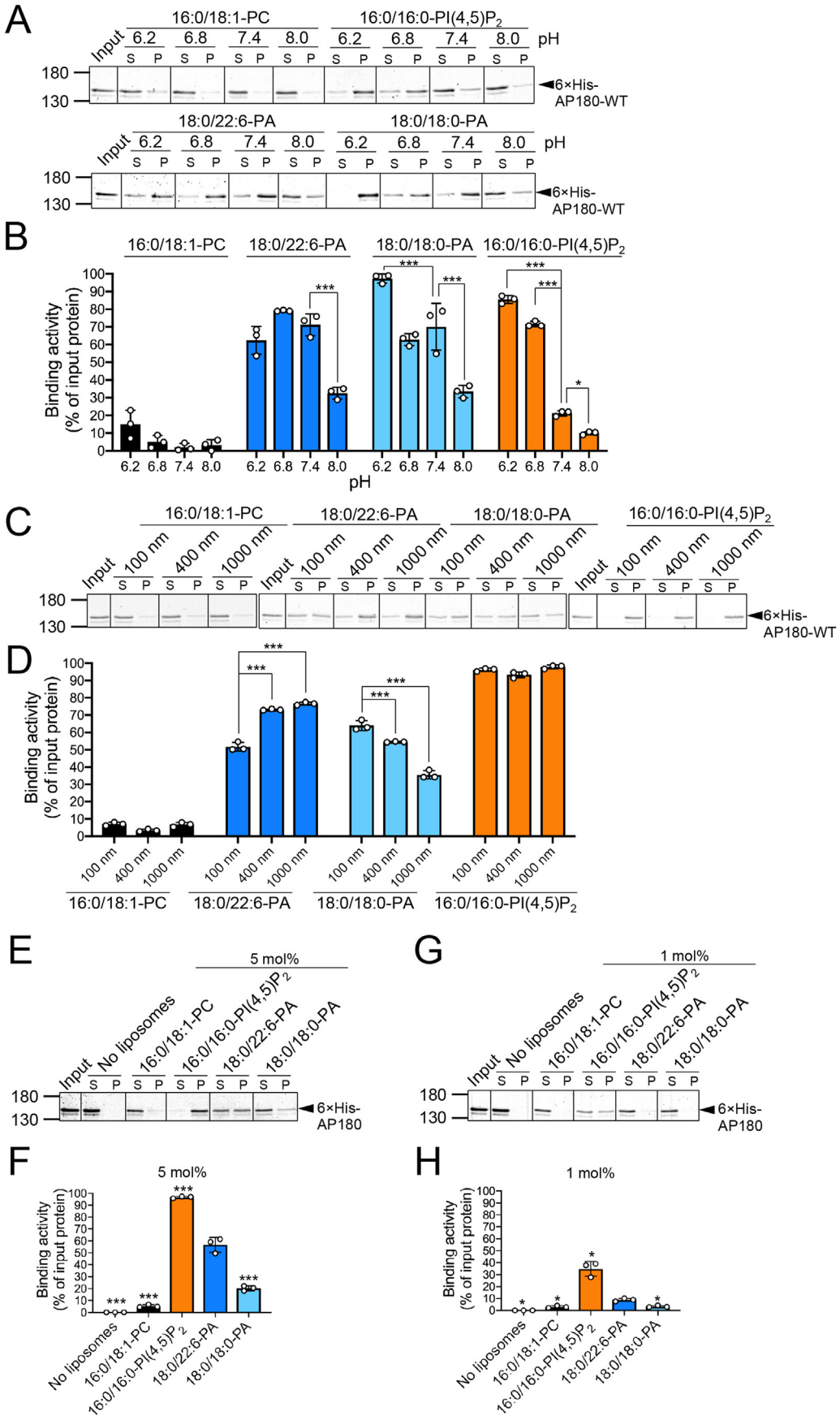
The diameter of clathrin-coated vesicles is 60–120 nm [24]. We next performed liposome binding assays using liposomes with different diameters of 100 nm, 400 nm or 1000 nm. As shown in Fig. 3C and D, AP180 strongly bound to PI(4,5)P_2 independent of liposome diameter. The binding activities of 18:0/18:0-PA and 18:0/22:6-PA were moderately affected by liposome diameters.

The concentrations of PI(4,5)P_2 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 PI(4,5)P_2. Approximately 95% and ~30% of AP180 interacted with 5 mol% (Fig. 3E and F) and 1 mol% (Fig. 3G and H) PI(4,5)P_2-containing liposomes, respectively. Moreover, AP180 showed ~55% and ~10% binding activities to 5 mol % (Fig. 3E and F) and 1 mol% (Fig. 3G and H) 18:0/22:6-PA-containing liposomes. These results indicate that AP180 can bind to PI(4,5)P_2 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

Fig. 2. 18:0/18:0-, 18:0/22:6-PA and PI(4,5)P_2 bound to AP180 via ANTH. (A and B) The 6×His-ANTH (1–289 aa), 6×His-CID (290–901 aa) and 6×His-AP180-KE (Lys38/39/40Glu) proteins expressed in *E. coli* cells were purified, separated by SDS–PAGE (10% acrylamide), stained with *CBB* or detected by *WB* with anti-His tag antibody (B). Liposome binding assay of (C and D) 6×His-ANTH, (E and F) 6×His-CID and (G and H) 6×His-AP180-KE using 16:0/18:1-PC-, 16:0/16:0-PI(4,5)P_2-, 18:0/18:0-PA- or 18:0/22:6-PA-liposomes (X/PC/chol = 10/60/30 mol% (X = PC, PA or PI(4,5)P_2)) (1 mM total lipid). The purified (C) 6×His-ANTH, (E) 6×His-CID or (G) 6×His-AP180-KE (0.2 μM) was incubated with each liposome (1 mM total lipids) and then separated by ultracentrifugation. SDS–PAGE (12 or 6% acrylamide) was performed, and separated proteins were stained with *CBB*. The amounts of protein in the supernatant (*S*) and precipitate (*P*) were quantified by densitometry using ImageJ software (D, F and H). 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.


A
16:0/18:1-PC
16:0/16:0-PI(4,5)P2
Input
6.2
6.8
7.4
8.0
pH
S P
180
130
6×His-AP180-WT
18:0/22:6-PA
18:0/18:0-PA
B
16:0/18:1-PC
18:0/22:6-PA
18:0/18:0-PA
16:0/16:0-PI(4,5)P2
Binding activity (% of input protein)
pH
C
100 nm
400 nm
1000 nm
D
E
5 mol%
No liposomes
16:0/18:1-PC
16:0/16:0-PI(4,5)P2
18:0/22:6-PA
18:0/18:0-PA
6×His-AP180
F
5 mol%
G
1 mol%
H
1 mol%

with PI(4,5)P_2 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-KE 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-KE, which lacks 18:0/22:6-PA- and PI(4,5)P_2-binding activities (Fig. 2G and H), was used (Fig. 4B and C). Moreover, the reducing effect of PI(4,5)P_2 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 PI(4,5)P_2 (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 PI(4,5)P_2, functions as a selective and effective attenuator for the interaction of AP180 with clathrin (Fig. 4). Therefore, although 18:0/22:6-PA and PI(4,5)P_2 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 PI(4,5)P_2.

The pK_a values of D4- and D5-phosphate of PI(4,5)P_2 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 PI(4,5)P_2 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 PI(4,5)P_2, maintain strong interactions with AP180 in the pH range in which CME occurs.

AP180 interacted with PI(4,5)P_2-containing liposomes independent of their diameter (100–1000 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 PI(4,5)P_2 (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 PI(4,5)P_2-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 PI(4,5)P_2 and thus that the physiological effects of 18:0/22:6-PA on AP180 functions are comparable to or higher than those of PI(4,5)P_2 in neuronal calls.

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 DGKδ selectively produced 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 PI(4,5)P_2 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 PI(4,5)P_2, 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.

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Fig. 3. Characterization of the interaction between AP180 and PA. (A and B) Purified 6×His-AP180 was dissolved in HEPES (25 mM HEPES, 100 mM NaCl, 1 mM DTT at pH 8.0 and 7.4) or MES (100 mM MES, 100 mM NaCl, 1 mM DTT at pH 6.8 and 6.2) buffer and incubated with 16:0/18:1-PC-, 16:0/16:0-PI(4,5)P_2-, 18:0/18:0-PA- or 18:0/22:6-PA-liposomes (X/PC/chol = 10/60/30 mol% (X = PC, PA or PI(4,5)P_2)) (1 mM total lipid) and then separated by ultracentrifugation. (C and D) Liposome binding assay of 6×His-AP180 using liposomes with different diameters of 100 nm, 400 nm or 1000 nm produced by a Mini Extruder was performed using the same experimental procedures described in Fig. 1D–G. (E and F) Liposome binding assay of 6×His-AP180 using 16:0/18:1-PC-, 16:0/16:0-PI(4,5)P_2-, 18:0/18:0-PA- or 18:0/22:6-PA-liposomes (X/PC/chol = 5/65/30 mol% (X = PC, PA or PI(4,5)P_2)) and (G and H) (X/PC/chol = 1/69/30 mol% (X = PC, PA or PI(4,5)P_2)) (1 mM total lipid) and then separated by ultracentrifugation. SDS/PAGE (6% acrylamide) was performed, and separated proteins were stained with *CBB* (C, E and G). The amounts of 6×His-AP180 in the supernatant and precipitate were quantified by densitometry using ImageJ software (D, F and H). 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.

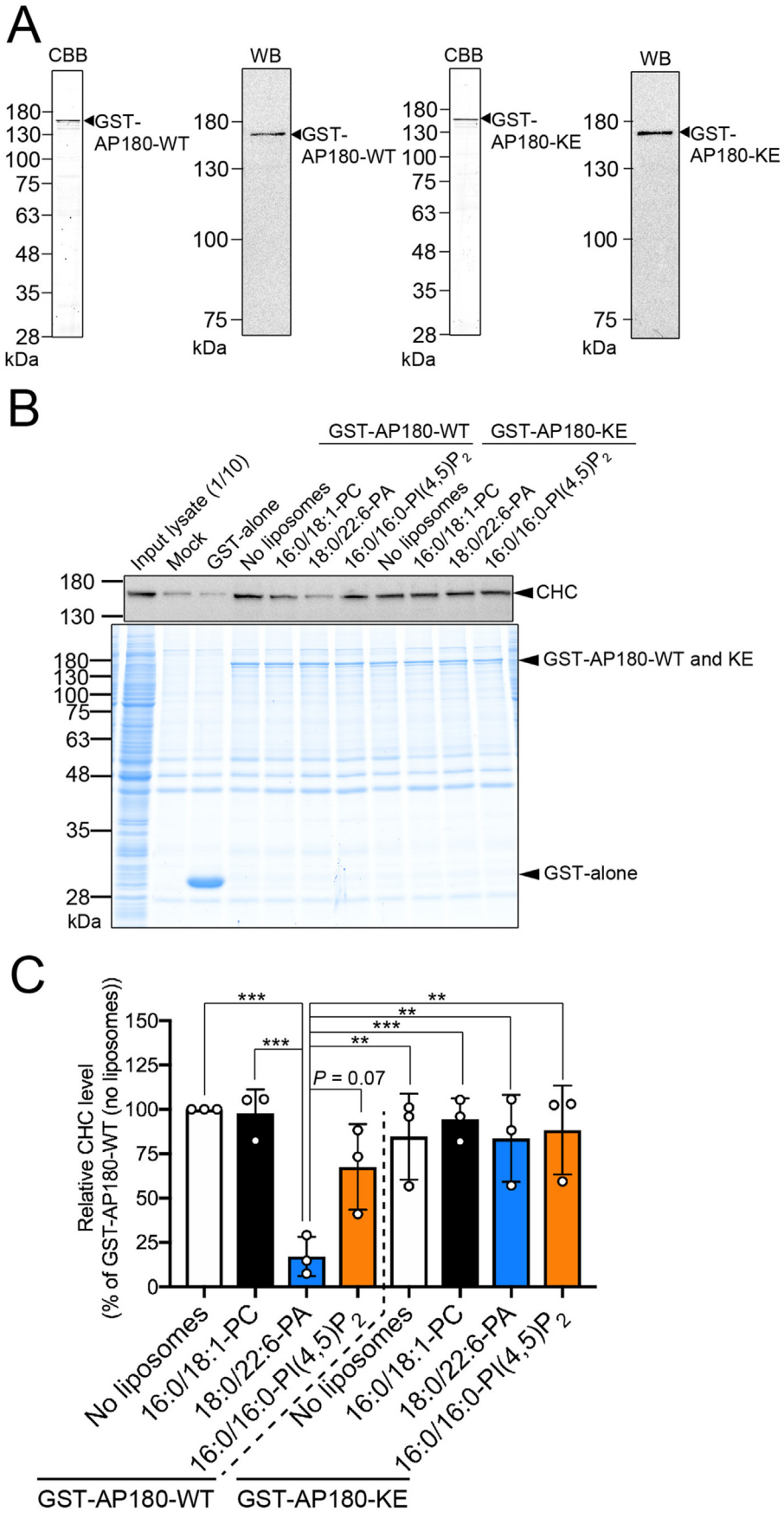
A
CBB
WB
CBB
WB
180
130
100
75
63
48
35
28
kDa
GST-AP180-WT
GST-AP180-KE
B
GST-AP180-WT
GST-AP180-KE
Input lysate (1/10)
Mock
GST-alone
No liposomes
16:0/18:1-PC
18:0/22:6-PA
16:0/16:0-PI(4,5)P2
CHC
GST-AP180-WT and KE
GST-alone
C
Relative CHC level
(% of GST-AP180-WT (no liposomes))
150
125
100
75
50
25
0
***
**
P = 0.07
No liposomes
16:0/18:1-PC
18:0/22:6-PA
16:0/16:0-PI(4,5)P2
GST-AP180-WT
GST-AP180-KE

Fig. 4. 18:0/22:6-PA inhibits the interaction between clathrin and AP180. (A) The GST-AP180-WT and GST-AP180-KE (Lys38/39/40Glu) proteins expressed in *E. coli* cells were purified, separated by SDS–PAGE (6% acrylamide), stained with *CBB* 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 glutathione-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-PI(4,5)P_2- or 18:0/22:6-PA-liposomes (X/PC/chol = 10/60/30 mol% (X = PC, PA or PI(4,5)P_2)) (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 *CBB* and analyzed by *WB* using anti-CHC antibody. (C) The amounts of CHC in the 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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