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Physiological Roles of Clathrin Adaptor AP Complexes: Lessons from Mutant Animals

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Clathrin-associated adaptor protein (AP) complexes play a key role in the transport of proteins, by regulating the formation of transport vesicles as well as cargo selection, between organelles of the post-Golgi network, namely, the *trans*-Golgi network (TGN), endosomes, lysosomes and the plasma membrane. Evidence has been accumulating for the physiological importance of AP complexes. Deficiency in AP-1A or AP-2 results in embryonic lethality in mice, indicating that these AP complexes are essential for normal development of embryos in mammals. In contrast, mutations in the genes encoding subunits of AP-3A cause an autosomal recessive disorder, Hermansky-Pudlak syndrome in human and its disease models in mice. Knockout mice for the neuron-specific AP-3B suffer from epileptic seizure. Further studies on the physiological and pathological aspects of AP complexes will not only be beneficial for better understanding of developmental biology and medical sciences, but also deepen our insight into the molecular mechanisms of vesicular traffic.

Key words: adaptor protein (AP) complex, clathrin, gene targeting, mutation, vesicular transport.

Protein transport among the organelles of secretory and endocytic systems is mediated in general by small, membrane-bound transport vesicles, and the process is thus termed vesicular transport (*1*). Accumulating evidence has underscored the physiological importance of vesicular transport in multicellular organisms, not to mention its essential roles as a fundamental cellular function.

Transport vesicles are classified by the identity of the protein coat used in their formation and also by the cargo they contain. Of those, clathrin-coated vesicles (CCVs) are responsible for the transport of proteins between organelles of the post-Golgi network, namely, the *trans*-Golgi network (TGN), endosomes, lysosomes and the plasma membrane (*2, 3*). Clathrin is a large heterohexameric protein complex composed of three heavy chains and three light chains (*4, 5*). Clathrin molecules self-assemble together to make a spherical "clathrin lattice" structure, a polyhedron made of regular pentagons and hexagons. The clathrin lattice serves as a mechanical scaffold but is itself unable to bind directly to membrane components. The connection of the clathrin scaffold to the membrane is mediated by clathrin adaptors, which can bind directly to both the clathrin lattice and to the lipid and protein components of membranes (*4, 6*). Clathrin-associated adaptor protein (AP) complexes are a stoichiometric coat component of CCVs alongside clathrin itself, and are considered a major clathrin adaptor contributing the CCV formation (*2–4, 6*).

In the past decade, evidence has been accumulating for the physiological importance of AP complexes. It is these studies that are the main focus of this article. The molecular basis for the function of AP complexes will also be briefly overviewed. For a more detailed description of the molecular mechanisms of action for AP complexes, please refer to other review articles (*2, 3, 6, 7*).

AP complexes regulate post-Golgi vesicular traffic

The AP complexes AP-1, AP-2, AP-3, and AP-4 play key roles in transport vesicle formation and cargo selection in post-Golgi trafficking pathways (*2, 3, 6, 8*) (Fig. 1). In addition to these ubiquitously expressed complexes, AP-1 and AP-3 have cell-type–specific isoforms: the epithelium-specific AP-1B and the neuron-restricted AP-3B. To distinguish them, the ubiquitous isoforms are designated as AP-1A and AP-3A. Interestingly, *Drosophila melanogaster* (*D. melanogaster*) and *Caenorhabditis elegans* (*C. elegans*) have single gene encoding a hybrid b1/2 protein that is probably shared by AP-1 and AP-2 (*8*).

Each AP complex consists of four subunits: two large (α, γ, δ or ε, and β1-4), one medium (μ1-4) and one small (σ1-4). Each of these subunits fulfills a specific function within the complex. One of the large subunits in each AP complex (α, γ, δ or ε) mediates binding to the target membrane (*9*). The other large subunit (β1-3) recruits clathrin through a clathrin-binding sequence, the clathrin box, in the hinge region (*10*). Although β4 lacks the clathrin box sequence, a morphological study has

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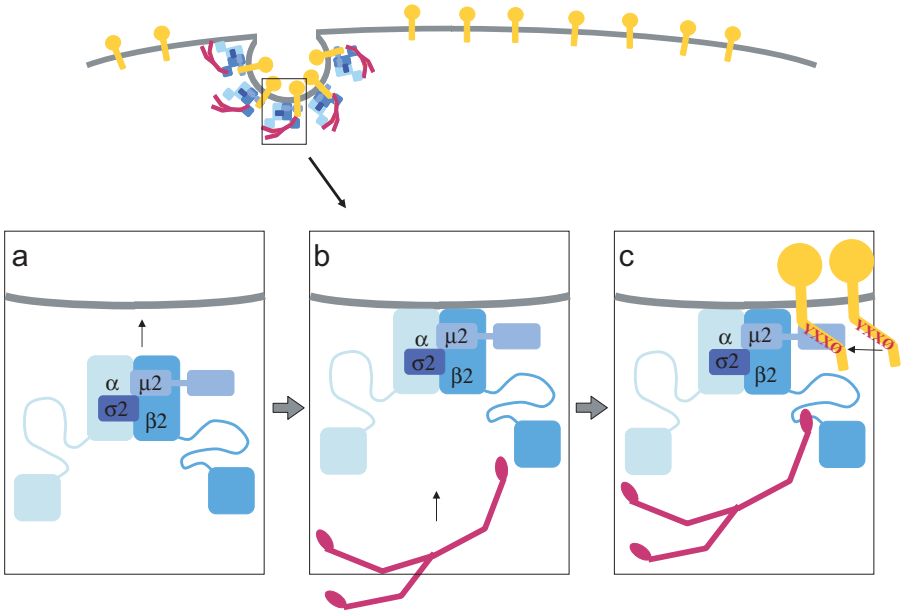

Fig. 1. **Schematic representation of the mechanism of action for the AP complex.** Mode of action for the AP complex is depicted by using AP-2 as the representative. AP-2 regulates the formation of CCV and selection of cargo. CCV formation initiates when AP-2 is recruited from the cytosol to the plasma membrane, mainly by the affinity of the a subunit with the lipid components of the membrane (a). Next, clathrin is recruited, again from the cytosol, to the membrane-bound AP-2 (b). Upon binding to AP complexes, clathrin can self-assemble to form the clathrin lattice which serves as a mechanical scaffold to bend the membrane. Transmembrane cargo proteins are thought to move relatively freely by diffusion in the membrane. When they diffuse into forming CCVs, they are trapped by coat component to be concentrated for selective sorting. Cargo proteins containing the YXXØ-type sorting signal in their cytoplasmic region are directly recognized and bound by the C-terminal domain of m2 (c).

suggested AP-4 could also interact with clathrin (*11*). In contrast, the μ subunits are involved in cargo selection by directly recognizing, with its C-terminal domain, the YXXØ-type (Y is tyrosine, X is any amino acid and Ø is a bulky hydrophobic amino acid) tyrosine-based-sorting signals present in the cytoplasmic domains of transmembrane proteins, thus mediating the selective capture of these proteins into CCVs (*12, 13*). The small subunits are thought to be involved in the stabilization of the complex (*9*). The N-terminal domains of μ subunits show a certain degree of homology with σ subunits and are thought to share the stabilizing role of the complex (*8*).

Each AP complex is involved in distinct vesicular transport pathways in the post-Golgi system. AP-2 mediates the formation of CCVs from the plasma membrane for endocytosis, which are destined for fusion with the early endosome (*9, 14*). AP-2 also serves as a cargo receptor to selectively sort the membrane proteins involved in receptor-mediated endocytosis, such as transferrion receptors (TfnR) in the forming CCVs (*12*). AP-1A, in conjunction with GGA, regulates the vesicular transport of cargos such as mannose 6-phosphate receptors (MPRs) between TGN and endosomes, although it is still unclear in which direction they operate (*6*). AP-1B, the epithelium-restricted isoform, is involved in polarized sorting of cargos such as TfnR and low-density lipoprotein receptor (LDLR) to the basolateral plasma membrane in polarized epithelial cells (*15–17*). AP-3A is believed to traffic cargo from an early endosomal compartment to the late endosomes/multivesicular bodies (MVBs) and/or lysosomes and lysosome-related organelles (*2, 6*). Studies on a neuroendocrine cell line PC12 have implicated the neuron-specific AP-3B in the biogenesis of synaptic vesicles from endosomes (*18, 19*). Consistent with this notion, AP-3B is preferentially concentrated to neuronal processes in primary culture of neurons (*20*). AP-4 has been reported to mediate the transport of some lysosomal proteins from TGN to lysosomes (*21*). It has also been proposed to be involved in basolateral transport of LDLR in a polarized MDCK epithelial cell line (*22*).

AP-1A is essential for embryonal development in mice

Two different AP-1A–deficient mice have been reported: KO mice for genes encoding γ (*23*) and μ1A (*24*) subunits (Table 1). Both are embryonic lethal, but the two mutant mice display distinct phenotypes. γ-KO mice die before day 4.5 postcoitus (E4.5) blastocyst stage (*23*). At E2.5 of mouse development, translation from maternal mRNA fades, while transcription of zygotic mRNA starts (*25*). Apparently, development of γ-KO embryos ceases when the amount of the γ subunit declines below a threshold because of the lack of embryonal γ subunit products. It seems likely, therefore, that AP-1 is essential for cell viability. It is also possible that AP-1 is not necessary for cell survival itself, but rather required for a more complicated function as a multicellular system such as development of embryos beyond the blastocyst stage or nidation. In this sense, successful establishment of embryonic fibroblasts from μ1A-KO embryos (*24*) (see below for details) may be inconsistent with the notion that AP-1A is essential for cell viability. Embryonic lethality of γ-KO mice also implies that the absence of γ cannot be compensated for by the γ2 protein, which shares 60% indentity with γ and is expressed ubiquitously (*26*).

In contrast to γ-KO mice, μ1A-KO embryos survive until E13.5 (*24*). Although no direct evidence is available, it seems likely that the presence of the μ1B isoform could compensate, at least in part, for the absence of μ1A, which likely explains the difference in timing of death observed between the two KO genotypes. Expression of μ1B is restricted to epithelial cells and the epidermal tissue in mice at E16 embryos as well as in adult (*15*) (and our unpublished observation). Epithelial cells are predominant in embryos at the earlier stages of development. When organogenesis proceeds to generate organs without μ1B

Table 1. **Genetic analyses of the function of adaptins and related proteins in multicellular organisms.**

Complex	Organism	Cause of the mutation	Phenotype
AP-1			
γ	*M. musculus*	gene targeting	embryonic lethal before E4.5
μ1A	*M. musculus*	gene targeting	embryonic lethal at E13.5, hemorrhage in the CNS
γ	*C. elegans*	dsRNAi	embryonic lethal
β1/2	*C. elegans*	dsRNAi	embryonic lethal
μ1 (*unc-101*)	*C. elegans*	natural mutant, dsRNAi	~50% larval lethal, uncoordinated movement
μ1 (*apm-1*)	*C. elegans*	dsRNAi	larval lethality
σ1	*C. elegans*	dsRNAi	embryonic lethal
AP-2			
μ2	*M. musculus*	gene targeting	embryonic lethal before E3.5
α	*D. melanogaster*	P-element insertion	embryonic lethal (in case of the loss-of-function allele)
α	*C. elegans*	dsRNAi	embryonic lethal
μ2	*C. elegans*	dsRNAi	embryonic lethal
σ2	*C. elegans*	dsRNAi	embryonic lethal
AP-3			
β3A	*H. sapiens*	natural mutant (HPS-2)	pigmentation defect, bleeding disorder, misrouting of lysosomal membrane proteins to the plasma membrane
δ	*M. musculus*	natural mutant (*mocha*)	pigmentation defect, bleeding disorder, lysosomal abnormalities, inner ear disorder, hyperactivity, abnormal electrocorticogram
β3A	*M. musculus*	natural mutant (*pearl*), gene targeting	pigmentation and bleeding defects
μ3B	*M. musculus*	gene targeting	epileptic seizure, impaired neurotransmission
δ	*D. melanogaster*	natural mutant (*garnet*)	pigmentation defect
β3	*D. melanogaster*	natural mutant (*ruby*)	Like *garnet*
μ3	*D. melanogaster*	natural mutant (*carmine*)	Like *garnet*
σ3	*D. melanogaster*	natural mutant (*orange*)	Like *garnet*

expression such as the brain, heart and liver, the lack of μ1B may cause cell death and/or malfunction in these organs and eventually lead to embryonic lethality. This notion is supported by the fact that the hemorrhage into the ventricle of brain and the spinal canal is seen as early as E10 in μ1A-KO mice (*24*). It should be noted that ES cells also express μ1B (our unpublished observation), which may aid survival at the earliest stages of μ1A-deficient embryos.

In accordance, μ1B could partially rescue the impairment in intracellular transport of membrane proteins observed in embryonic fibroblasts derived from μ1A-KO mouse embryos (*27*). In fibroblasts lacking μ1A, normal localization of MPRs and furin at the TGN disappears and, instead, they mislocalize to the endosomal compartment (*24*). When μ1B is exogenously expressed in these fibroblasts, TGN localization of MPRs can be recovered, whereas furin stays mislocalized in endosomes (*27*).

In *C. elegans*, there exist two m1 genes for AP-1, *unc-101* and *apm-1* (*28*). Unlike the mammalian homologues, however, the μ1 isoforms are expressed ubiquitously throughout development. Disruption of the two μ1 genes simultaneously causes embryonic lethality, whereas single disruptions of either one have distinct phenotypes (*28, 29*), indicating that the two isoforms may have distinct functions. RNAi of any one of the other three subunits of AP-1, as well as the simultaneous disruption of *unc-101* and *apm-1*, results in embryonic lethality. This suggests that the two μ1 chains may share the other three subunits.

AP-2–deficient mice also suffer from early embryonic lethality

Despite the detailed characterization of the molecular mechanisms of AP-2 function, its physiological role has not been directly assessed until recently. Studies with cultured cells using dominant-negative (*30–32*) and RNA interference (RNAi) (*33–37*) approaches have shown that AP-2 is indeed required for the rapid internalization of the transferrin receptor as well as some lysosome-associated membrane proteins (LAMPs) that traffic via the plasma membrane. A notable finding from these studies is that depletion of AP-2 by RNAi of cultured cells did not cause apparent loss of viability over the limited time span of the experiments. However, RNAi-treated cells still contained small amounts of residual AP-2, which could have been sufficient to sustain cell viability. Meanwhile, in vivo function of AP-2 has been studied in *C. elegans* (*38, 39*) and *D. melanogaster* (*40*). These studies showed that AP-2 plays a critical role in embryonic development.

To assess the requirement for AP-2 in the context of a whole mammalian organism, we carried out a targeted disruption of the gene encoding μ2 in mouse. We found that the heterozygous μ2 mutant mice are viable and phenotypically normal, but the homozygous mutant embryos die before the E3.5 blastocyst stage (*41*). The most likely explanation for the early death of μ2-deficient embryos is that maternally inherited μ2 mRNA becomes too dilute at the stage of cleavage division around E3.5, as is the case for γ1-deficient embryos. We also failed to obtain $\mu2^{-/-}$ ES cells in culture (unpublished observation). Taken together,

these observations suggest that the AP-2 complex is indispensable for cell viability. It is thus likely that the small amount of μ2 remaining in the RNAi-treated cells still allows for the maintenance of cell viability. A similar observation has been made for clathrin. RNAi knockdown of clathrin heavy chain drastically inhibits transferrin receptor endocytosis but apparently does not lead to cell death (*33–37, 42*). In contrast, ablation of the clathrin heavy chain gene in the chicken DT40 B-cell line leads to apoptotic cell death (*43*).

Mutation of AP-3 causes Hermansky-Pudlak syndrome

Hermansky-Pudlak syndrome (HPS) consists of a group of genetically different autosomal recessive disorders which share the clinical findings of oculocutaneous albinism, a platelet storage pool deficiency, and some degree of ceroid lipofuscinosis (*44, 45*). These symptoms appear because the function and/or biogenesis of lysosomes and lysosome-related organelles such as melanosomes and platelet dense granules are impaired. One of the HPS-causing genes, *AP3B1*, has been found to encode the β3A subunit of AP-3A complex (*46*), whose mutations result in a subtype of HPS called HPS-2. β3A cDNA contains 3950 bases and is translated into 1094 amino acid β3A protein. Two brothers with HPS-2 were reported to display compound heterozygous mutations in *AP3B1* consisting of a 21 amino acid deletion (D390–410) and a single amino acid substitution, L580R, due to a T to G substitution. Subsequently, a third patient with HPS-2 was reported to have compound heterozygous nonsense mutations in *AP3B1*: C1578T (R to X) resulting in termination at codon 509, and G2028T (E to X) resulting in termination at codon 659 (*47*). As a result, Western blot analysis detected little or no β3A protein in the skin fibroblasts isolated from the patients. These fibroblasts displayed enhanced trafficking of LAMPs through the plasma membrane en route to lysosomes, which is consistent with a role for AP-3A in protein trafficking from endosomes to lysosomes and the importance of AP-3A in biogenesis/function of lysosomes and lysosome-related organelles.

In mouse, at least 16 mutants are known as models for HPS (*48*). Among them, mutations for *pearl* and *mocha* have been identified in the genes encoding β3A (*49*) and δ (*50*) subunits, respectively, of the AP-3 complex. These mice and HPS-2 patients share the same clinical symptoms such as coat color dilution and prolonged bleeding time. In addition, fibroblasts isolated from these mice also display the abnormally enhanced expression of LAMPs on the plasma membrane. β3A-null mice generated by gene targeting technique showed a similar phenotype to *pearl* mice (*51*), confirming that β3A mutation is responsible for the HPS-2/Pearl phenotype.

The AP-3 δ subunit is shared by ubiquitous AP-3A and neuron-restricted AP-3B. As a reflection of this fact, *mocha* mice, which lack both AP-3A and AP-3B, additionally suffer from neurological abnormalities (*50*). These include inner ear disorder, namely, impaired balance and hearing loss (*52*), as well as behavioral hyperactivity and abnormal electrocorticogram (*53*). A phenotypically mild allele of *mocha, mh*^{2j}, has also been reported, which results in less diluted coat color and infrequent inner ear disorder, but more frequent tonic-clonic behavioral seizures that are rare in *mocha* mice (*54*).

D. melanogaster and *C. elegans* possess a single AP-3 isoform which is ubiquitously expressed (*8, 55*). Consistent with the pigmentation defect in AP-3A deficient mammals described above, deficiencies of AP-3 subunits in *D. melanogaster* were found to correspond to eye color mutants, in which the delivery of proteins to pigment granules was impaired (*56–59*). In contrast to the nonlethal phenotype of AP-3A deficiency in mammals and *D. melanogaster*, RNAi of genes encoding ubiquitous AP-3 subunits in *C. elegans* causes embryonic and larval lethality (*55*). The molecular basis for this discrepancy is currently unknown.

AP-3B deficiency causes epileptic seizure in mice

Since neurological abnormalities are restricted to *mocha* (*50*) but not seen in *pearl* (*49*), these findings raised the possibility that the neurological abnormalities observed in *mocha* mice are due to the absence of neuron-specific AP-3B. Alternatively, the neurological phenotypes may appear only when both AP-3A and AP-3B are missing simultaneously. To clarify this problem, we have generated and analyzed the mice deficient in μ3B, a neuron-specific subunit of AP-3B (*60*). μ3B-KO mice were fertile and survived for at least one year. Although μ3B-KO mice appeared normal, some adult mice exhibited spontaneous epileptic seizures upon presentation of such stimuli as positional change. Seizure susceptibility of μ3B-KO mice was confirmed by pharmacological as well as electrical stimulation. Morphological abnormalities in both excitatory and inhibitory synapses as well as synaptic vesicles were observed in these mice. Biochemical studies demonstrated the impairment in release of the inhibitory neurotransmitter γ-aminobutyric acid (GABA), which likely resulted, at least in part, from the reduction of vesicular GABA transporter (VGAT) in μ3B-KO mice, which facilitated the induction of long-term potentiation and the abnormal propagation of neuronal excitability in the hippocampus. Thus, μ3B plays a critical role in the normal formation and function of a subset(s) of synaptic vesicles, which is likely reflected in the impairment in inhibitory GABAergic neurons. This could cause the imbalance in excitatory and inhibitory neuronal activities, and ultimately lead to recurrent epileptic seizure.

In contrast to epileptic seizure, μ3B-KO mice exhibit neither deafness nor impaired balance. The inner ear disorder has been attributed to an insufficiency of heavy metals such as zinc and/or manganese (*52*). Kantheti *et al.* (*50*) reported the lack of zinc as well as ZnT-3, a zinc transporter localized in synaptic vesicles, in the brain of *mocha* mice. By contrast, we observed neither any apparent differences in zinc staining nor the immunolocalization of ZnT-3 in the hippocampus of μ3B-KO mice or *pearl* mice (our unpublished observation), consistent with the lack of inner ear symptoms in these mice. These observations suggest that the inner ear phenotype as well as the mislocalization of ZnT-3 appears only when both AP-3A and AP-3B are deficient in *mocha* mice. Recently, another mutant mice for AP-3B, β3B-KO mice have been described (*20*). These mice are phenotypically

similar to μ3B-KO mice in that they are hyperactive and suffer from tonic-clonic seizures. However, thsy display a slight decrease in the amount of histochemically reactive zinc in the cortex and hippocampus and of ZnT-3 in synaptic vesicle preparation, although the inner ear problem has not been reported in these mice. It is possible that the difference in genes defective in these two mice strains contributes to the distinct phenotypes observed, even though the two gene products assemble together to make the neuron-specific AP-3B complex. Whether the decrease of zinc and ZnT-3 in β3B-KO mice is physiologically significant remains to be seen.

Perspectives

As described above, great advances have been achieved to reveal that AP complexes play important roles in the physiology and pathology of multicellular organisms. Nevertheless, many questions remain to be answered. The role of AP-4 is still ambiguous, not only at the physiological level but also at the molecular level. AP-4 is conserved in mammals, chicken and the plant *Arabidopsis thaliana*, but not in *D. melanogaster* or *C. elegans* (*8*), which suggests a sophisticated function of AP-4 in higher eukaryotes. Also unsolved is the molecular basis of the discrepancy observed in AP-3 deficiency: a nonlethal phenotype in mammals as well as in fruit fly, but embryonic lethal phenotype in nematode. Concerning the epithelium-specific AP-1B, we have recently established m1B-deficient mice and observed abnormal morphology in intestinal epithelium (manuscript in preparation). Further studies on the physiological and pathological aspects of AP complexes will not only afford a better understanding of developmental biology and medical sciences, but also deepen our insight into the molecular mechanisms of vesicular traffic.

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