Neuronal and non-neuronal functions of the AP-3 sorting machinery

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Summary

Vesicles selectively exchange lipids, membrane proteins and luminal contents between organelles along the exocytic and endocytic routes. The repertoire of membrane proteins present in these vesicles is crucial for their targeting and function. Vesicle composition is determined at the time of their biogenesis by cytosolic coats. The heterotetrameric protein adaptor protein complex 3 (AP-3), a coat component, participates in the generation of a diverse group of secretory organelles and lysosome-related organelles. Recent work has shed light on the mechanisms that regulate AP-3 and the trafficking pathways controlled by this adaptor. Phenotypic analysis of organisms carrying genetic deficiencies in the AP-3 pathway highlight its role regulating the targeting of lysosomal, melanosomal and synaptic vesicle-specific membrane proteins. Synaptic vesicles from AP-3-deficient mice possess altered levels of neurotransmitter and ion transporters, molecules that ultimately define the type and amount of neurotransmitter stored in these vesicles. These findings reveal a complex picture of how AP-3 functions in multiple tissues, including neuronal tissue, and expose potential links between endocytic sorting mechanisms and the pathogenesis of psychiatric disorders such as schizophrenia.

Key words: AP-3, BLOC-1, Dysbindin, Schizophrenia, Mental disorder, Hermansky-Pudlak, Zinc transporter, Synaptic vesicle, ZnT3

Introduction

Membrane-enclosed organelles possess distinctive protein compositions dynamically maintained by vesicle formation and vesicle fusion mechanisms (Bonifacino and Glick, 2004). These vesicles are generated by cytosolic coat proteins that selectively concentrate specific membrane proteins into departing vesicles (Bonifacino and Glick, 2004). Heterotetrameric coat protein complexes known as adaptor protein complexes (AP-1, AP-2, AP-3 and AP-4) act as scaffolds bringing together membrane lipids, sorting signals present in the cytosolic domains of membrane proteins, components of the vesicle fusion machinery and additional components of the vesicle formation apparatus (Boehm and Bonifacino, 2001; Robinson, 2004; Sorkin, 2004). The function of these adaptors is linked to the donor organelle in which they perform their sorting function. AP-1 generates vesicles from the trans-Golgi network that transport cargoes bound for late-endosome and lysosome compartments, whereas AP-1 adaptors found in endosomes generate vesicles routed to the cell surface or back to the Golgi complex. AP-2 resides exclusively in the plasma membrane, where it generates endocytic vesicles. AP-3 is present in endosomes and delivers proteins to late endosomes and lysosomes. In neurons, AP-3 generates synaptic vesicles or vesicles carrying synaptic vesicle membrane proteins. Finally, AP-4 is localized to the trans-Golgi network, where it produces vesicles that transport specific lysosomal proteins (Ohno, 2006; Robinson, 2004). In each case, at the donor compartments, adaptors cycle between cytosolic and membrane-bound pools, and this process is controlled by inositol phospholipids (Balla and Balla, 2006; Di Paolo and De Camilli, 2006) and ARF GTPases (Robinson, 2004).

In this commentary, we concentrate on the function, regulation and interactions of the vertebrate adaptor AP-3 from the perspective of neuronal membrane protein traffic. Recent studies offer the potential to further expand our understanding of effects associated with loss, dysfunction or subversion of the AP-3 machinery – for example, in the generation of HIV particles (Dong et al., 2005), and possibly in the pathogenesis of schizophrenia (Norton et al., 2006; Owen et al., 2005) and other psychiatric disorders (Breen et al., 2006). Discussion of insights into the AP-3 route gained from studies of invertebrate systems and budding yeast can be found elsewhere (Boehm and Bonifacino, 2002; Lloyd et al., 1998; Odorizzi et al., 1998).

Genetics of the AP-3 pathway in vertebrates

Much of our knowledge about AP-3 function in metazoans comes from the phenotypic analysis of mouse and human AP-3 mutations as well as mutations in gene products that interact with this adaptor complex (Di Pietro and Dell'Angelica, 2005; Li et al., 2004). Deficiencies in at least 14 loci, including AP-3 gene defects, trigger a syndrome characterized by systemic and in some cases neurological phenotypes. The systemic defects include occulo-cutaneous pigment dilution, platelet

dysfunction, pulmonary fibrosis (Di Pietro and Dell'Angelica, 2005; Li et al., 2004), recurrent infections due to defects of innate immunity (Fontana et al., 2006; Jung et al., 2006), cyclic neutropenia (Benson et al., 2003) and cytotoxic T lymphocyte (CTL) defects (Clark et al., 2003). These systemic phenotypes define in humans the Hermansky-Pudlak Syndrome (HPS1 to HPS8, OMIM 203300), a disease generated by defects in at least eight genetic loci (Di Pietro and Dell'Angelica, 2005; Li et al., 2004). HPS disorder type 2 (HPS2) specifically results from altered AP-3 function in humans (Clark et al., 2003; Dell'Angelica et al., 1999; Fontana et al., 2006; Jung et al., 2006; Sugita et al., 2002). Epilepsy and hyperactivity characterize the neurological symptoms found in a group of these genetic deficiencies (Kantheti et al., 2003; Kantheti et al., 1998; Nakatsu et al., 2004; Seong et al., 2005) as well as arthrogryposis due to motor axon defects (Gissen et al., 2004).

The majority of these 14 genes encode polypeptides that assemble into five protein complexes: AP-3 (Fig. 1), BLOC-1 to BLOC-3 (Fig. 5) (biogenesis of lysosome-related organelles complex), and HOPS (homotypic vacuolar protein sorting or VPS class C complex) (Di Pietro and Dell'Angelica, 2005). These complexes are present in endosomes, where they participate in membrane protein sorting and vesicle biogenesis (Di Pietro et al., 2006; Peden et al., 2004; Theos et al., 2005). Vesicles generated by the AP-3–BLOC machinery carry membrane proteins bound to a wide range of secretory organelles, such as lysosomes, lysosome-related organelles (e.g. melanosomes, platelet dense granules, azurophilic granules and surfactant granules) (Di Pietro and Dell'Angelica, 2005; Li et al., 2004; Wei, 2006) and synaptic vesicles (Nakatsu et al., 2004; Salazar et al., 2004b; Seong et al., 2005). This diversity of organelles regulated by AP-3–BLOC complexes explains the pleiotropic nature of the phenotypes already described.

A

Gene Name	Protein Name	Expression	Mutants
Ap3d	δ	Ubiquitous	*mocha*
Ap3b1	β3A	Ubiquitous	HPS2, *pearl*, *Ap3b1-/-*
Ap3b2	β3B	Neuronal	*Ap3b2-/-*
Ap3m1	μ3A	Ubiquitous	
Ap3m2	μ3B	Neuronal	*Ap3m2-/-*
Ap3s1	σ3A	Ubiquitous	
Ap3s2	σ3B	Ubiquitous	

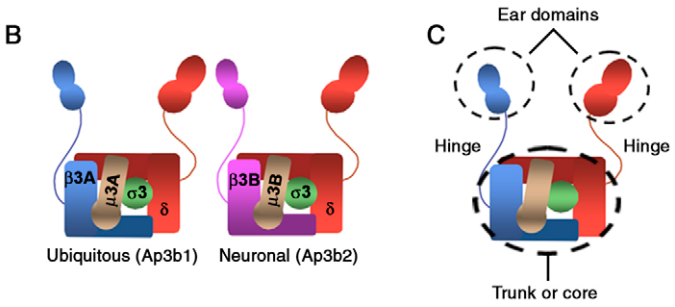

Fig. 1. Nomenclature and structure of AP-3 subunit isoforms. (A) AP-3 subunit genes and their corresponding gene products. The pattern of expression of each subunit is described as neuronal, corresponding to neurons and neuro-endocrine tissue, or ubiquitous, for subunits expressed in all cells including neuronal tissues. Mice carrying natural or engineered AP-3 subunit deficiencies are listed. (B) Proposed subunit composition of the neuronal and ubiquitous AP-3 isoforms. Both AP-3 complexes can carry either a σ3A or a σ3B subunit. (C) Adaptor complexes possess three defined domains. The ears correspond to the C-terminal domains of δ and β3. The trunk or core is composed of a protease-resistant core formed by fragments of β3 and δ as well as full-length μ3 and σ3 subunits. The ears and trunk are connected by hinges.

Mutations affecting vertebrate AP-3 subunits

AP-3 consists of four subunits δ, β3, μ3 and σ3. β3, μ3 and σ3 each exist as two isoforms (A and B; Fig. 1A,B) (Boehm and Bonifacino, 2001; Boehm and Bonifacino, 2002; Robinson, 2004). Two of these isoforms, β3B (Darnell et al., 1991; Gurkan et al., 2005; Newman et al., 1995) and μ3B (Gurkan et al., 2005; Pevsner et al., 1994), are largely restricted to neuronal and neuroendocrine tissues, whereas both σ3A and σ3B are ubiquitously expressed (Fig. 1A,B) (Dell'Angelica et al., 1997a; Gurkan et al., 2005). β3B and μ3B are thought to assemble into neuronal isoform complexes containing δ and σ3A or σ3B subunits (Fig. 1B). By contrast, β3A, μ3A, δ, and σ3A or σ3B subunits are part of the ubiquitous AP-3 adaptor isoform present in all cells, including neurons (Fig. 1B). Murine deficiencies in four of these AP-3 subunits (δ, β3A, β3B and μ3B; Fig. 1A) (Feng et al., 1999; Kantheti et al., 1998; Nakatsu et al., 2004; Seong et al., 2005; Yang et al., 2000) recapitulate to different degrees the phenotypes that result from the most severe form of AP-3 deficiency, the *mocha* mutation (Kantheti et al., 1998; Lane and Deol, 1974; Noebels and Sidman, 1989). Systemic phenotypes and neurological alterations characterize the *mocha* allele in mice (Kantheti et al., 2003; Kantheti et al., 1998; Lane and Deol, 1974; Miller et al., 1999; Noebels and Sidman, 1989; Rolfsen and Erway, 1984). Mocha mice lack the δ subunit of AP-3, a subunit common to all AP-3 complexes (Kantheti et al., 1998). The consequence of the mocha defect is the degradation of all neuronal and ubiquitous AP-3 subunits (Kantheti et al., 1998; Peden et al., 2002). By contrast, β3A genetic deficiencies (*pearl* and *Ap3b1*$^{-/-}$ in mice; HPS2 in humans; Fig. 1A) elicit degradation of the ubiquitous AP-3 (Peden et al., 2002; Yang et al., 2000; Zhen et al., 1999). β3A mutations lead to systemic phenotypes (Yang et al., 2000; Zhen et al., 1999).

Regulation of AP-3 function

The mechanisms controlling recruitment of AP-3 to membranes and AP-3-dependent membrane protein sorting include GTPases (Dell'Angelica et al., 1997a; Faundez et al., 1997; Faundez et al., 1998; Ooi et al., 1998; Simpson et al., 1996), kinases (Faundez and Kelly, 2000; Salazar et al., 2005b), intermediate filament proteins (Styers et al., 2006; Styers et al., 2004), accessory proteins (Crump et al., 2001), and clathrin (Dell'Angelica et al., 1998) (Fig. 2). Best understood is the regulated recruitment of AP-3 to membranes by Arf GTPases. These GTPases cycle between GTP- and GDP-bound forms, controlling recruitment and release of coat proteins from membranes. Brefeldin A interferes with this cycle, leading to accumulation of GDP-Arf1, and thus prevents the binding of coats such as AP-3 to membranes (Jackson and Casanova, 2000; Zeghouf et al., 2005; Dell'Angelica et al., 1997a; Drake et al., 2000; Simpson et al., 1996). Similarly, Arf1 mutants locked in their GDP-bound form prevent the binding of AP-3 to organelles (Faundez et al., 1998; Ooi et al., 1998). By contrast, an Arf1 mutant unable to hydrolyse GTP holds AP-3 on membranes (Faundez et al., 1998; Ooi et al., 1998).

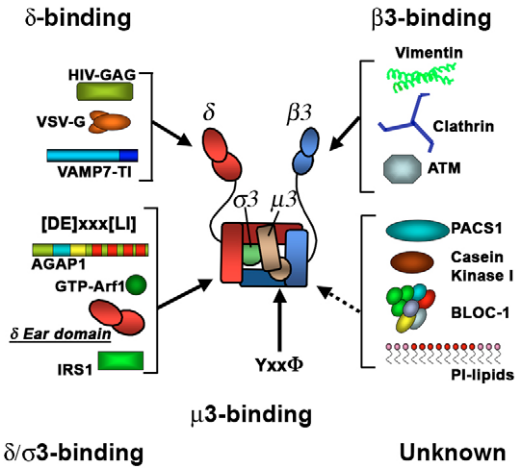

Fig. 2. Molecular interactions of the adaptor complex AP-3. The diagram depicts AP-3 subunits. δ is shown in red, σ3 is shown in green, μ3 is shown in brown and β3 is shown in blue. Known interactions with a particular AP-3 subunit are indicated by a solid arrow. Interactions with AP-3 where the subunit is not known are depicted by a dashed arrow. These interactions correspond to PACS-1 (Crump et al., 2001), a casein kinase (Faundez and Kelly, 2000), BLOC-1 (Di Pietro et al., 2006) and inositol phospholipids (PI-Lipids). Vimentin (Styers et al., 2004), clathrin (Dell'Angelica et al., 1998) and the ataxia telangiectasia gene product (ATM) (Lim et al., 1998) directly associate with the β3 subunit. The δ subunit provides a platform sufficient to bind HIV Gag protein (Dong et al., 2005), protein G of the vesicular stomatitis virus (VSV-G) (Nishimura et al., 2002) or the R-(v)- SNARE VAMP7-TI (Martinez-Arca et al., 2003). Alternatively δ adaptin in a complex with σ3 or σ3 by itself binds di-leucine sorting motifs ([DE]xxx[LI]) (Janvier et al., 2003), AGAP1 (Nie et al., 2003), the Arf1 GTPase (Lefrancois et al., 2004), the δ ear domain (Lefrancois et al., 2004), and the insulin receptor substrate 1 (IRS1) (VanRenterghem et al., 1998). Tyrosine sorting motifs (YxxΦ) bind to the μ3 subunits (Ohno et al., 1995).

GTP-bound Arf1 directly interacts with the AP-3 complex (Austin et al., 2002; Lefrancois et al., 2004). The association involves the σ3 subunit or the δ-σ3 AP-3 subcomplex (Fig. 2) (Austin et al., 2002; Lefrancois et al., 2004). The crystal structure of the adaptor complexes AP-1 and AP-2 reveals that their σ subunits are buried in the tetramer (Collins et al., 2002; Heldwein et al., 2004). Thus, the σ3-Arf1 interaction suggests that, in AP-3, σ3 becomes exposed partially or transitorily to the cytosolic environment. Notably, the interaction is selective since Arf5 does not appear to interact with AP-3 (Austin et al., 2002). Moreover, a GDP-locked Arf5 mutant does not affect recruitment of AP-3 to membranes (Ooi et al., 1998). These results suggest that AP-3 directly discriminates between different Arf GTPases and/or that factors affecting Arf1 GTPase activity are selectively recognized by AP-3.

The intrinsic GTPase activity of Arf1 is very low and nucleotide hydrolysis only occurs in the presence of members of a diverse family of GTPase activating proteins (ArfGAPs) (Nie and Randazzo, 2006). AGAP1 is an ArfGAP that selectively affects Arf-dependent recruitment of AP-3 to membranes. AGAP1 contains four types of domain, among them Arf GAP and pleckstrin homology (PH) domains (Nie et al., 2003). The latter can bind inositol phospholipids (Balla, 2005) and in AGAP1 it binds to the AP-3 δ-σ3 subcomplex (Nie et al., 2003) (Fig. 2). The AGAP1 PH domain could thus functionally link inositol phospholipids with adaptor complexes. AGAP1 GAP activity is stimulated by phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] and phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3] in vitro (Nie et al., 2002). Thus, AGAP1 could regulate the levels of membrane-bound AP-3 by sensing membrane concentrations of PtdIns(4,5)P_2 and/or PtdIns(3,4,5)P_3. Key to understanding Arf1-AGAP1-mediated regulation of AP-3 recruitment is, therefore, the identification of the enzymes that generate inositol phospholipid species and the mechanisms by which such enzymes reach and/or define domains of AP-3 vesicle formation.

One such potential enzyme, phosphatidylinositol 4-kinase type II alpha (PI4KIIa), phosphorylates phosphatidylinositol (PtdIns) to generate phosphatidylinositol (4)-phosphate, a precursor of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (Balla and Balla, 2006). PI4KIIa is present in endosomes (Balla et al., 2002; Guo et al., 2003; Minogue et al., 2006; Salazar et al., 2005b) and is a major component of AP-3-derived vesicles (Salazar et al., 2005b). The kinase is targeted to vesicles by an AP-3-dependent mechanism, which suggests that it behaves as an AP-3 cargo (Salazar et al., 2005b). However, the kinase is not limited to acting as a passive cargo. Its expression affects the subcellular localization of AP-3 to endosomes: downregulation of the kinase leads to a redistribution of endosomal AP-3 to cytosol (Salazar et al., 2005b); conversely, overexpression of the kinase increases AP-3-dependent vesicle generation (Salazar et al., 2005b).

How are PI4KIIa, Arf1, AGAP1, and AP-3 integrated to generate vesicles? GTP-Arf1-dependent recruitment of AP-3 to membranes should be one of the primary events. We envisage that, once on membranes, AP-3 binds PI4KIIa and concentrates this enzyme into endosomal domains able to generate vesicles. In these domains, inositol phospholipids generated by PI4KIIa should further recruit AP-3 and increase the activity of AGAP1. Accumulation of coat and cargo in a nascent vesicle depends on recruitment of an inactive AGAP1 by AP-3. Thus, only when PI4KIIa lipid products reach a particular threshold level, would AGAP1 be activated and coat recruitment finish. This model assumes that binding AP-3 to AGAP1 decreases its Arf1GAP activity and holds it in an inactive state until enough lipid species have been produced to activate its GAP activity. This is the case for the closely related ArfGAP AGAP2, whose GAP activity towards Arf1 is inhibited by the presence of the adaptor complex AP-1, to which AGAP2 selectively binds (Nie et al., 2005).

PI4KIIa and AGAP1 represent extrinsic factors capable of regulating recruitment of AP-3 to membranes. However, AP-3 has an intrinsic mechanism that can regulate its recruitment to membranes. The AP-3 δ ear domain (see Fig. 1C) binds to the σ3 subunit of AP-3 (Fig. 2), preventing the binding of GTP-Arf1 to AP-3 and recruitment of the adaptor to membranes (Lefrancois et al., 2004). An intramolecular regulatory mechanism could thus couple binding of AP-3 to membranes with Arf1 levels and nucleotide status. Feedback mechanisms involving the enzymatic activities of Arf1 and PI4KIIa may further control the type of cargo and the coat content in nascent AP-3 vesicles; these remain to be explored.

The elusive role of clathrin in AP-3 vesiculation

Arf1 has an indisputable function in the early stages of formation of AP-3 vesicles. However, clathrin's role at later stages remains unclear. Clathrin provides a scaffold to bind adaptors, and accessory factors involved in cargo recognition, membrane curvature and uncoating (Conner and Schmid, 2003; Kirchhausen, 2000; Traub, 2005). Formation of AP-1 and AP-2 vesicles requires clathrin both in vivo and in vitro (Conner and Schmid, 2003; Hinrichsen et al., 2006; Kirchhausen, 2000; Miwako et al., 2003; Pagano et al., 2004; Traub, 2005). By contrast, in vivo and in vitro data on the role of clathrin in AP-3 vesicle formation are conflicting.

In vitro AP-3-dependent sorting proceeds in the absence of clathrin (Faundez et al., 1997; Faundez et al., 1998) and in the presence of clathrin-perturbing reagents (Shi et al., 1998), which indicates that clathrin may not be necessary for formation of AP-3 vesicles. Indeed, AP-3 is absent from purified clathrin-coated vesicles from brain (Blondeau et al., 2004; Dell'Angelica et al., 1997b; Simpson et al., 1996), and vesicles partially coated with AP-3 lack detectable levels of clathrin (Salazar et al., 2005b). Collectively, this evidence suggests that clathrin is dispensable for AP-3 vesicle formation. However, it could also indicate that clathrin plays cargo-selective roles in AP-3-dependent sorting rather than vesicle formation. In addition, the absence of AP-3 in clathrin-coated vesicles may just reflect labile clathrin–AP-3 interactions that do not withstand the purification procedure or the relative levels of clathrin–AP-3 coated vesicles in different cell types. In fact, immunoelectron microscopy identifies clathrin-decorated, AP-3 budding profiles on endosomes (Dell'Angelica et al., 1998; Peden et al., 2004; Theos et al., 2005), and AP-3 is present in clathrin-coated vesicles isolated from HeLa (Borner et al., 2006) and PC12 cells (G. Salazar and V.F., unpublished data). Furthermore, clathrin binds to AP-3 in cell-free conditions through the β3 subunit of AP-3 (Dell'Angelica et al., 1998) (Fig. 2). The crystal structure of the β3 clathrin-binding motif bound to the N-terminal domain of clathrin shows that the binding mechanism is similar to that of other clathrin-binding proteins (ter Haar et al., 2000) and uses a conserved 'clathrin-box' motif (Dell'Angelica, 2001; ter Haar et al., 2000). However, β3 mutants lacking the clathrin-box motif still rescue a characteristic missorting phenotype in β3-deficient fibroblasts (Peden et al., 2002), which challenges the idea that clathrin has a role in AP-3 function.

A caveat of such studies is that they only assess the effects on one cargo protein (Peden et al., 2002; Shi et al., 1998). Lack of the clathrin box could lead, for example, to selective sorting alterations rather than defective vesicle formation itself. Alternatively, the clathrin box in β3 subunits may recruit clathrin in vitro but not be necessary for clathrin recruitment in vivo. Indeed, only half of all endosomal AP-3 budding profiles are decorated with clathrin in immunoelectron microscopy experiments (Dell'Angelica et al., 1998; Peden et al., 2004; Theos et al., 2005). Thus, the clathrin box may not be functional in some AP-3 complexes, or an AP-3-interacting protein may selectively recruit clathrin to certain AP-3 vesicle buds. Irrespective of the mechanism, this suggests that AP-3 complexes generate vesicles that have different compositions.

Not all AP-3s are created equal: neuronal versus ubiquitous adaptors

AP-3 complexes assembled by neuronal AP-3 adaptin isoforms could generate vesicles with different cargo composition. This idea is strengthened by the observation that μ3A and μ3B subunits present in ubiquitous and neuronal AP-3 complexes recognize different tyrosine-based sorting motifs (Ohno et al., 1998). Because of their restricted expression in brain tissue, β3B and μ3B subunits are assumed to assemble exclusively into neuronal AP-3 complexes, whereas β3A and μ3A assemble into a ubiquitously expressed complex (Fig. 1B). This notion is supported by the observation that β3B protein levels are reduced in μ3B-deficient mouse brain (Nakatsu et al., 2004). However, analysis of the neuronal phenotypes observed in β3B-deficient (*Ap3b2*$^{-/-}$) and μ3B-deficient (*Ap3m2*$^{-/-}$) mice suggests a more complex picture. If neuronal AP-3 subunit isoforms were to assemble exclusively into neuronal complexes, then targeted disruption of either the *Ap3b2* or the *Ap3m2* locus in the same background should lead to identical brain phenotypes. In fact, although both mouse mutants display juvenile spontaneous epilepsy (Nakatsu et al., 2004; Seong et al., 2005) they have different zinc transporter (ZnT3) phenotypes.

ZnT3 is a metal transporter exclusively targeted to synaptic vesicles and is necessary for luminal storage of ionic zinc in synaptic vesicles (Cole et al., 1999; Palmiter et al., 1996; Salazar et al., 2004b). Synaptic vesicle ZnT3 levels are normal in μ3B-deficient mice but reduced in β3B-deficient mice (Nakatsu et al., 2004; Seong et al., 2005). This suggests partially divergent functions of AP-3 complexes assembled with β3B or μ3B. Differences in the phenotypes of β3B-deficient and μ3B-deficient mice may be due to partially overlapping expression of these gene products in different brain regions – as suggested by EST expression profiles in UNIGENE and the membrome database (Gurkan et al., 2005; Pevsner et al., 1994) (http://symatlas.gnf.org/SymAtlas/). Alternatively, the remaining β3B in *Ap3m2*$^{-/-}$ mice could form δ-σ3-β3B-μ3A tetramers, which could account for their lack of a zinc transporter phenotype.

Heterologous expression of neuronal AP-3 adaptin isoforms in non-neuronal cells indicates that neuronal subunits can assemble into complexes containing ubiquitous AP-3 isoforms (Peden et al., 2002). β3A and the neuronal β3B isoform effectively rescue the phenotypes observed in β3A-deficient fibroblasts, which demonstrates that the endogenous μ3A isoforms present in these non-neuronal cells forms complexes with both β3 subunits (Peden et al., 2002). Therefore, the spectrum of AP-3 complexes assembled in neuronal tissue could even be more diverse than presumed. These considerations are important in the interpretation of the phenotypes caused by loss of AP-3 in the brain.

The function(s) of AP-3 complexes in neuronal cells

The hypothesis that AP-3 adaptors are involved in the sorting of synaptic membrane proteins emerged after the identification of AP-3 μ transcripts in the electric lobe of the ray *Discopyge ommata*, a tissue rich in synaptic terminals (Pevsner et al., 1994). The staining of synaptic terminals by human anti-β3B autoimmune antibodies supported this idea (Newman et al., 1995). However, it was not until the development of cell-free vesicle biogenesis assays that definitive evidence implicated

AP-3 in targeting of synaptic vesicle membrane proteins (Blumstein et al., 2001; Faundez et al., 1998; Shi et al., 1998). In these assays targeting of proteins requires either AP-3-containing brain cytosol or purified brain AP-3 and ARF1 (Blumstein et al., 2001; Faundez et al., 1998; Shi et al., 1998).

The sorting of synaptic vesicle membrane proteins is controlled by AP-3 in vivo (Fig. 3), as illustrated by the cellular phenotypes of the *Ap3d*$^{-/-}$ (*mocha*), *Ap3b2*$^{-/-}$, and *Ap3m2*$^{-/-}$ mice. The *mocha* strain is characterized by epilepsy and hyperactivity (Kantheti et al., 2003; Kantheti et al., 1998; Lane and Deol, 1974; Miller et al., 1999; Noebels and Sidman, 1989; Rolfsen and Erway, 1984). Their nerve terminals lack ionic zinc owing to defective sorting of ZnT3 (Kantheti et al., 2003; Kantheti et al., 1998; Salazar et al., 2004b; Stoltenberg et al., 2004) which binds directly and selectively to AP-3 (Salazar et al., 2004b). The defective targeting of ZnT3 in *mocha* and *Ap3b2*$^{-/-}$ (Seong et al., 2005) mouse models as well as the defective GABAergic neurotransmission in *Ap3m2*$^{-/-}$ mice (Nakatsu et al., 2004) provide conclusive evidence that synaptic vesicle targeting defects are part of the AP-3-deficient neuronal phenotype.

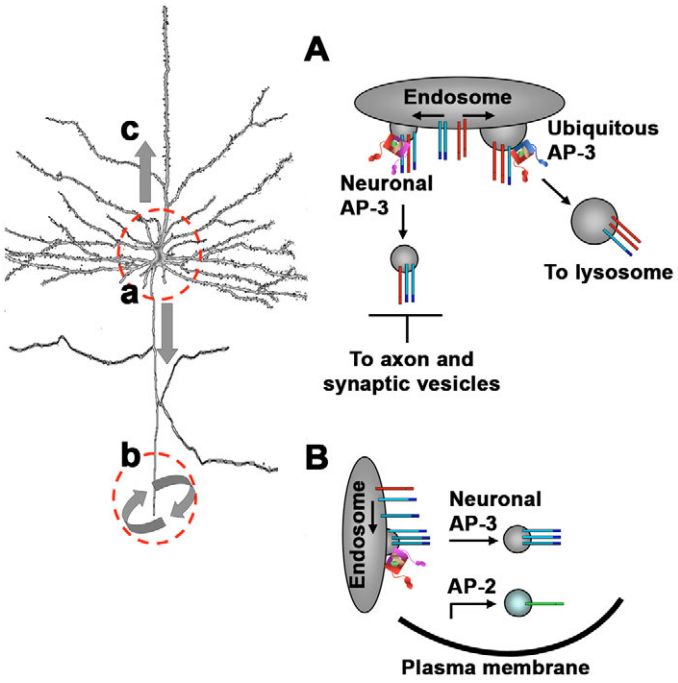

Fig. 3. AP-3 sorting mechanisms in neuronal cells. A neuron possesses neuronal and ubiquitous AP-3 complexes in the cell body (a) but only neuronal AP-3 in axons (b) and dendrites (c) (Seong et al., 2005). (A) Proposed functions of the neuronal and ubiquitous AP-3 in cell bodies. Both AP-3 forms reside in the same endosome. Neuronal AP-3 sorts proteins into the axon or a synaptic vesicle pathway. Ubiquitous AP-3 sorts proteins to the lysosomal pathway. Model A explains the changes in ZnT3 levels observed in *Ap3b1*$^{-/-}$ and *Ap3b2*$^{-/-}$ mice (Seong et al., 2005). In this model AP-3-sorted proteins, like ZnT3, are recognized by both AP-3 isoforms and therefore routed to either a synaptic vesicle-axonal or a lysosomal pathway. Thus, in the absence of one AP-3 isoform, membrane proteins are targeted to the other pathway. The amount of ZnT3 in synaptic vesicles is reduced in the absence of neuronal AP-3 (*Ap3b2*$^{-/-}$) and ZnT3 is instead routed to lysosomes for degradation. Similarly, the amount of ZnT3 targeted to lysosomes is reduced in the absence of ubiquitous AP-3 (*Ap3b1*$^{-/-}$) and it is then routed by the neuronal AP-3 (encoded by *Ap3b2*) to synaptic vesicles, thus triggering increased levels of ZnT3 in synaptic vesicles. Model B shows the proposed role of neuronal AP-3 in synaptic vesicle biogenesis in presynaptic terminals. AP-3 generates synaptic vesicles from presynaptic endosomes. This route is parallel to the AP-2 route, which generates synaptic vesicles from the plasma membrane. A role for AP-3 in sorting to dendrites (c) has not been documented yet.

Several other synaptic vesicle membrane proteins are mistargeted in AP-3-deficient brains. These include synaptic vesicle chloride channel 3 (ClC-3) (Salazar et al., 2004a), vesicular glutamate transporter 1 (VGLUT1) (Salazar et al., 2005a), PI4KIIa (Salazar et al., 2005b), the vesicular GABA transporter (VGAT) (Nakatsu et al., 2004), and the synaptobrevin-1-like SNARE VAMP7-TI (also known as Sybl1, VAMP7 or TI-VAMP) (Salazar et al., 2006; Scheuber et al., 2006). Defective targeting of VAMP7 in *mocha* brain affects spontaneous and evoked release at hippocampal mossy fiber synapses (Scheuber et al., 2006). Similarly to ZnT3, several of these membrane proteins are sorted by AP-3 into endosome-derived microvesicles when expressed in PC12 cells. An important consideration is that these synaptic vesicle protein targeting defects are selective as evidenced by the normal levels of ubiquitous synaptic vesicle membrane proteins such as synaptophysin, the 116-kDa subunit of the vacuolar ATPase, and SV2 in vesicles (Salazar et al., 2005a; Salazar et al., 2006; Salazar et al., 2005b; Salazar et al., 2004a; Salazar et al., 2004b; Seong et al., 2005).

Despite the defective targeting of selected synaptic vesicle proteins, electrophysiological analysis of *mocha* brain hippocampus reveals that short-term synaptic plasticity and synaptic vesicle recycling remain unaffected (Vogt et al., 2000), although this involves only low-frequency stimulation. These observations are consistent with the preponderant role that AP-2 plays in synaptic vesicle biogenesis at the synapse (Fig. 3B) (Murthy and De Camilli, 2003). However, recent electrophysiological and neuronal imaging evidence indicate that AP-3 contributes to synaptic vesicle (Voglmaier et al., 2006) and large dense core vesicle biogenesis (Grabner et al., 2006). In contrast to the AP-2 pathway, AP-3-dependent synaptic vesicle sorting mechanisms contribute to synaptic vesicle recycling only under high-frequency stimulation (Voglmaier et al., 2006). High frequency stimulation of motoneurons induces the formation of endosomes in presynaptic terminals (Heuser and Reese, 1973), a compartment from where AP-3 forms vesicles in other neuronal and non-neuronal cell types (Fig. 3B).

The neuronal phenotypes of AP-3-deficient animals already discussed point to a synaptic defect (Fig. 3). However, it is important to consider that the synaptic defects observed in *mocha* neurons may also reflect defective trafficking processes outside presynaptic terminals. Three observations are consistent with the hypothesis that AP-3 performs extra-synaptic functions in neurons. First, AP-3 staining is equally intense in axonal and dendritic processes (Seong et al., 2005); AP-3 may therefore have yet unknown dendritic functions. Second, immature amphibian motor neurons display a constitutive axonal vesicle recycling mechanism sensitive to brefeldin A (Zakharenko et al., 1999), much like the biogenesis of AP-3-bearing vesicles from endosomes in PC12 cells (Faundez et al., 1997). This brefeldin-A-sensitive recycling

mechanism, although present in axons, is absent at the site of contact between the motor neuron and myoblasts in cultured preparations (Zakharenko et al., 1999). Moreover, *mocha* hippocampal neurons, despite their dramatic reduction in PI4KIIa levels in nerve terminals, have normal or increased levels of this kinase in cell bodies (Salazar et al., 2005b). Similar observations have been made of BLOC-1 complex subunits (see below) (Salazar et al., 2006) and VAMP7 (Scheuber et al., 2006). These results suggest that vesicles containing synaptic vesicle proteins may be generated by AP-3 from endosomes either in the axon or in the cell body (Fig. 3A), before targeting to presynaptic terminals.

Neuronal functions for the 'non-neuronal' AP-3 complexes

Because of the absence of all AP-3 isoforms in *mocha* mutants, the contribution of neuronal and ubiquitous AP-3 isoforms to the neuronal phenotypes observed cannot be discerned. In a simple model, AP-3 isoforms perform different sorting functions: ubiquitous AP-3 regulates the biogenesis of neuronal lysosomes whereas neuronal AP-3 exclusively trafficks synaptic vesicle proteins. This hypothesis was founded on the observation that mice selectively lacking neuronal AP-3 (*Ap3b2*$^{-/-}$ or *Ap3m2*$^{-/-}$) (Nakatsu et al., 2004; Seong et al., 2005) exhibit an epileptic phenotype but mice lacking the ubiquitous AP-3 isoform do not (*Ap3b1*$^{-/-}$ or *pearl*) (Feng et al., 1999; Yang et al., 2000). However, closer inspection of the Ap3b1$^{-/-}$ brains reveals a selective increase in the content of synaptic vesicle AP-3 cargoes (ZnT3, and ClC-3) in synaptic vesicle fractions (Seong et al., 2005). These changes are paralleled by an increase in histochemically reactive zinc in *Ap3b1*$^{-/-}$ hippocampus and cortex (Seong et al., 2005). To a certain extent, AP-3 complexes assembled with β3A or β3B may thus recognize similar cargoes. Moreover, these findings suggest that subtle neurological and behavioral phenotypes previously not appreciated may occur in β3A-deficient and BLOC-1-null mice.

How can β3A- or β3B-containing complexes generate different phenotypes if both contribute to the recognition of overlapping synaptic vesicle proteins? This could be explained by differences in the spectrum of synaptic vesicles recognized by complexes carrying β3A or β3B, the subcellular localization of adaptor complexes carrying β3A and β3B, and/or the fate of the vesicles generated by these adaptor complexes. Vesicles generated by β3A-containing complexes could target synaptic vesicle membrane proteins to degradative compartments (Fig. 3A). This hypothesis is consistent with the increased content of synaptic vesicle AP-3 cargoes in *Ap3b1*$^{-/-}$ mice (Seong et al., 2005) and predicts the existence of an AP-3-derived vesicle population bound for lysosomal compartments that contains both synaptic vesicle proteins and lysosomal AP-3 cargoes. Proteomic analysis of organelle fractions enriched in AP-3-derived microvesicles suggests that lysosomal and synaptic vesicle AP-3 cargoes coexist in these fractions (Salazar et al., 2005b) as well as in purified brain synaptic vesicles (Takamori et al., 2006). The lysosomal proteins present in AP-3 microvesicles include LAMP1, vps33b, the R-(v)-SNARE VAMP7-TI, and four subunits of the octameric BLOC-1 complex (pallidin, dysbindin/sandy, snapin and muted. Importantly, these proteins either directly and/or genetically interact with AP-3 (Di Pietro and Dell'Angelica, 2005).

Interactions between AP-3 and other Hermansky-Pudlak gene products

BLOC-1 and AP-3 complexes are both thought to participate in the transport of membrane proteins destined for lysosomes and lysosomes-related organelles (Di Pietro and Dell'Angelica, 2005; Li et al., 2004). Genetic and cell biological evidence support two models of AP-3 and BLOC-1 function. In the first, AP-3 and BLOC-1 complexes act together and interact to sort components into the same vesicle transport mechanism (vesicle A in Fig. 4). In a second non-exclusive mechanism, AP-3 and BLOC-1 act independently, at different locations in endosomes, sharing overlapping but distinct sets of cargo proteins and generating two different types of vesicle (vesicles A and B in Fig. 4). Both mechanisms are likely to exist in mammalian cells but operate to different extents depending on the cell type (Di Pietro et al., 2006; Gautam et al., 2006; Salazar et al., 2006).

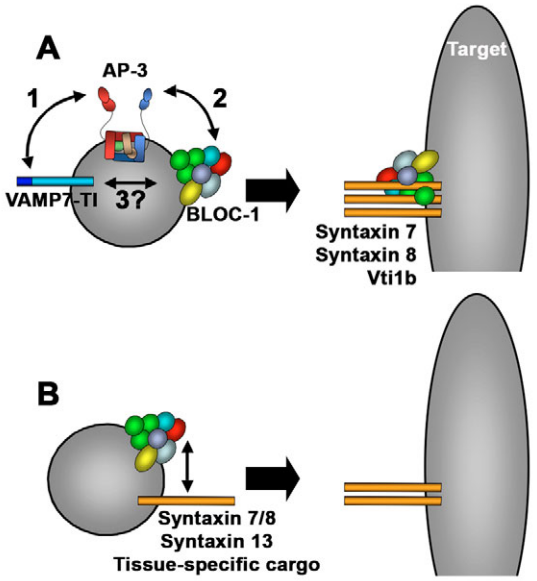

Fig. 4. Models of BLOC-1–AP-3 sorting functions. (A,B) Two different vesicles, A and B, fuse with a target organelle (right). VAMP7-TI is present in vesicle A (blue cylinder). Its cognate Q-(t)-SNAREs (syntaxin 7, 8 and Vti1b; orange cylinders) are present in the target organelle. Model A depicts AP-3 and BLOC-1 complexes co-residing in vesicle A (Salazar et al., 2006). In this vesicle, BLOC-1 may regulate the recognition of specific cargoes, like the SNARE VAMP7-TI, either by bridging AP-3 and a selected membrane protein (interactions 2 and 3) or by stabilizing specific AP-3–cargo interactions (interactions 1 and 2). Interactions 1 (Martinez-Arca et al., 2003) and 2 have been documented (Di Pietro et al., 2006) and (G. Salazar and V.F., unpublished results). Interaction 3 is speculative. On the target membrane a tripartite Q-(t)-SNARE complex of syntaxin 7, syntaxin 8, and Vti1b is maintained by BLOC-1, independently of AP-3. Model B depicts the selective sorting of SNAREs or tissue-specific cargo into vesicle B by the BLOC-1 complex. Vesicle B represents a vesicle population distinct from vesicle A. Sorting into and/or biogenesis of vesicle B requires BLOC-1 but not AP-3 function. Model A and model B are non-exclusive. Either Model A alone or a combination of models A and B explain both the convergent sorting phenotypes of BLOC-1 and AP-3 deficiencies as well as the altered colocalization between syntaxins 7 and 8, which is unique to BLOC-1–mutant cells (Salazar et al., 2006).

The notion that AP-3 and BLOC-1 act together is supported by the observed similarity of the cellular phenotypes of BLOC-1-deficient and AP-3-deficient skin fibroblasts. These phenotypes include: missorting of lysosomal proteins to the cell surface (CD63 and LAMP1) (Di Pietro et al., 2006; Salazar et al., 2006), mistargeting of PI4KIIa, and reduced levels of the R-(v)-SNARE VAMP7-TI (Salazar et al., 2006). Furthermore, cell biological studies reveal the presence of both complexes on the same AP-3 vesicle (Salazar et al., 2006) and interactions between AP-3 and BLOC-1 can be demonstrated by immunoprecipitation of an AP-3–BLOC-1 supra-complex either from isolated membranes (Di Pietro et al., 2006) or whole cells (G. Salazar and V.F., unpublished data) (Fig. 2).

The precise mechanism(s) by which the BLOC-1–AP-3 supra-complex regulates sorting into the same vesicle remains unknown (Fig. 4). BLOC-1 might play a role in recruitment of AP-3 to membranes (Di Pietro et al., 2006); however, this is unlikely to be at a rate-limiting step because the steady-state levels of membrane-bound AP-3 remain unaffected in BLOC-1-deficient cells (Salazar et al., 2006; Setty et al., 2007). Alternatively, BLOC-1 could recognize specific cargoes, such as SNAREs (Fig. 4A). Two subunits of the BLOC-1 complex, pallidin and snapin, can interact with the endocytic SNAREs syntaxin 13 (Huang et al., 1999; Moriyama and Bonifacino, 2002) and SNAP23-25 (Ilardi et al., 1999; Ruder et al., 2005; Tian et al., 2005), respectively (Fig. 5C). Furthermore, downregulation of another BLOC-1 subunit, dysbindin, decreases the levels of a subset of synaptic vesicle proteins, including the SNARE, SNAP25 (Numakawa et al., 2004). Consistent with these observations is the observation that the levels of the SNARE VAMP7-TI, an AP-3-interacting protein (Alberts et al., 2003; Martinez-Arca et al., 2003), are selectively reduced in AP-3-null and BLOC1$^{-/-}$ cells (Salazar et al., 2006).

It is likely, however, that BLOC-1 also functions in sorting independently of AP-3 (Fig. 4B). For example, BLOC-1, but not AP-3, deficiencies lead to reduced colocalization of the VAMP-7-binding cognate SNAREs syntaxin 7 and syntaxin 8 (Salazar et al., 2006), which allow VAMP-7-containing vesicles to fuse with late endosomes/lysosomes (Bogdanovic et al., 2002; Mullock et al., 2000; Pryor et al., 2004; Ward et al., 2000). The hypothesis that AP-3 and BLOC-1 act independently at different subcellular locations yet recognizing overlapping but distinct sets of cargo proteins is strongly supported by the observation that AP-3-null and BLOC-1-null double-knockout mice have phenotypes more pronounced than those observed in single-complex-deficient mice (Gautam et al., 2006).

The BLOC-1–AP-3 über-complex: a possible connection to schizophrenia

Despite similar functions of AP-3 and BLOC-1 in non-neuronal tissue, an important question is whether BLOC-1 and its interaction with AP-3 play a role in synaptic vesicle protein trafficking. Three lines of evidence implicate BLOC-1 in synaptic mechanisms. First, BLOC-1, like AP-3 complexes, is present in neuronal microvesicles containing synaptic vesicle markers as well as in hippocampal mossy fiber nerve terminals (Salazar et al., 2006; Talbot et al., 2006). Second, targeted disruption of snapin, a BLOC-1 subunit, leads to defective secretion of neurotransmitters in mice (Tian et al., 2005) (Fig. 5C). Finally, human genetic polymorphisms in the genes encoding the BLOC-1 subunits dysbindin, more commonly known in humans as dystrobrevin-binding protein 1 (DTNBP1) (Benson et al., 2004; Bray et al., 2005; Funke et al., 2004; Norton et al., 2006; Owen et al., 2005) and muted (Straub et al., 2005) may be associated with schizophrenia. However, the single patient deficient in dysbindin showed Hermansky-Pudlak syndrome without any reported mental illness (Li et al., 2003), and the *sandy* mouse lacking this protein has no yet reported behavioral phenotype (Li et al., 2003).

Defective presynaptic vesicle fusion has been hypothesized to be a pathogenic mechanism in schizophrenia (Camargo et al., 2007; Honer and Young, 2004; Mirnics et al., 2000). Schizophrenic brain possesses reduced levels of mRNA and/or proteins involved in synaptic vesicle fusion, such as NSF, VAMP2 and SNAP-25 (Halim et al., 2003; Honer et al., 2002; Knable et al., 2004; Mukaetova-Ladinska et al., 2002). In common with schizophrenia, AP-3 and BLOC-1 have been linked with the fusion machinery involved in synaptic vesicle secretion (Fig. 2 and Fig. 5C). Indeed, loss of AP-3 in the *mocha* mice affects spontaneous and evoked release at hippocampal mossy fiber synapses (Scheuber et al., 2006). Furthermore, recruitment of AP-3 to neuroendocrine

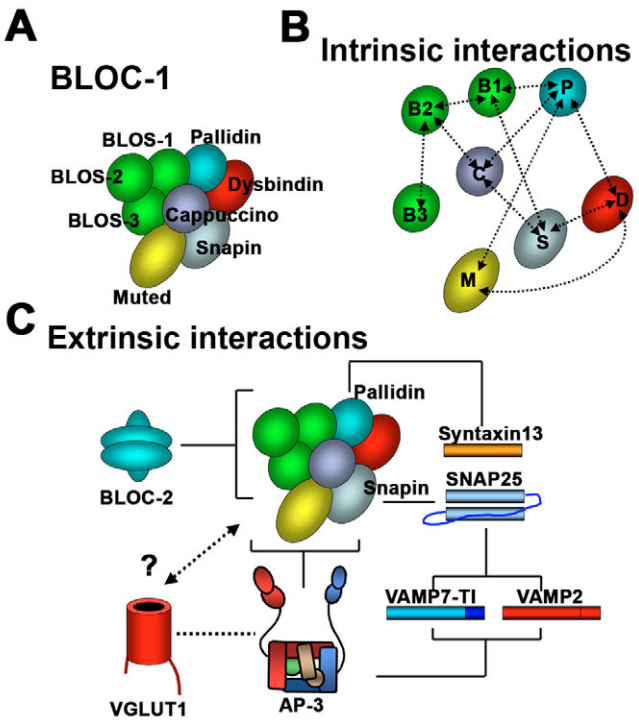

Fig. 5. Subunit composition and molecular interactions of the BLOC-1 complex. (A) The model depicts the known subunit structure of the BLOC-1 complex. (B) The name of each subunit is denoted by the initial letter of the protein name. The diagram describes the intrinsic interactions between subunits of the BLOC-1 complex. For details see (Di Pietro and Dell'Angelica, 2005). (C) The diagram represents a partial list of interactions (solid lines), functional relationships (dashed line), or potential relationships (double-headed arrow with question mark) relevant to vesicle generation, fusion, and the pathogenesis of schizophrenia. A detailed list of interactions is described in (Di Pietro and Dell'Angelica, 2005).

microvesicles is sensitive to tetanus neurotoxin (Salem et al., 1998). This agent selectively cleaves VAMP2 on synaptic vesicles and effectively halts synaptic vesicle fusion (Lalli et al., 2003). Furthermore, the BLOC-1 subunit snapin binds to SNAP-25, a Q-(t)-SNARE that forms a complex with VAMP2 (Tian et al., 2005) or VAMP7-TI (Alberts et al., 2003; Martinez-Arca et al., 2003) (Fig. 5C).

A role of AP-3 and BLOC-1 complexes in proper targeting of the synaptic vesicle fusion machinery is supported by the reduced levels of R-(v)-SNARE VAMP7-TI in both AP-3-deficient and BLOC-1-deficient mice (Salazar et al., 2006) its defective targeting to *mocha* nerve terminals (Scheuber et al., 2006), and the neurological phenotype of mice lacking the BLOC-1 subunit snapin. These mice possess impaired neurotransmitter secretion by mechanisms involving the SNARE SNAP-25 (Tian et al., 2005). The most compelling connection between the AP-3–BLOC-1 machinery and schizophrenia is the finding that brain tissue from schizophrenia patients has reduced levels of the BLOC-1 subunit dysbindin in hippocampal mossy fibers (Talbot et al., 2004). This phenotype is also found in AP-3-deficient *mocha* brain (Salazar et al., 2006). Finally, the presynaptic levels of the synaptic vesicle protein VGLUT1, which is sorted in part by AP-3 (Salazar et al., 2005a) (Fig. 5C), increase in hippocampal nerve terminals of schizophrenic patients (Talbot et al., 2004). A potential explanation for this is that its targeting to degradative compartments is impaired by the decreased levels of dysbindin.

Conclusions and perspectives

Although AP-3 mutations have helped us to identify multiple AP-3-dependent cellular functions, our understanding of the mechanisms that regulate AP-3 still lacks the molecular and structural detail of its predecessors, AP-1 and AP-2. The identification of the whole network of AP-3 interactors, regulators and adaptor accessory proteins remains a challenge. However, a potential list of these has recently been obtained (Salazar et al., 2005b). Definition of structural-functional modules in these proteins, as in many AP-1- and AP-2-interacting proteins, will greatly facilitate the dissection of the precise mechanisms that lead to the formation of AP-3 vesicles. Some of the components may behave as facultative molecules rather than be required for AP-3 function. This concept is particularly relevant considering that AP-3 might generate vesicles that have different compositions in the same cell.

A particularly intriguing problem that is common to several coat protein complexes is how isoforms contribute to the functional diversity of adaptors. We have begun to unravel this problem in the analysis of AP-1 (Folsch et al., 1999; Folsch et al., 2003; Folsch et al., 2001; Ohno et al., 1999; Sugimoto et al., 2002) and AP-3 adaptors (Nakatsu et al., 2004; Seong et al., 2005). However, the presence of two adaptor isoforms expressed in the same cell still leaves several unanswered questions regarding the regulation of assembly and function. Adaptors are assembled from shared polypeptides and tissue specific isoforms. For example, δ is common to neuronal and ubiquitous AP-3 (Fig. 1B). Thus, adaptors carrying a common subunit probably partially overlap and/or compete at the level of biosynthetic assembly, cargo or accessory polypeptide selection and subcellular distribution. In the search for common molecular mechanisms governing adaptor function, we have ignored the contribution that tissue-specific components may play a role in adaptor function. This is particularly clear in AP-3 and BLOC complex deficiencies, in which the phenotypes of double mutants are more pronounced than those of single mutants. However, the penetrance of the genetic interactions among AP-3 and BLOC complexes varies among tissues (Gautam et al., 2006). Furthermore, the δ subunit deficiencies *mocha* and the milder *mocha(2J)* differ in their zinc phenotypes. *Mocha* mice show a severe reduction of vesicular zinc in hippocampus and cortex. By contrast, *mocha(2J)* mice exhibit normal expression in hippocampal mossy fibers, but abnormal patterns in the neocortex (Kantheti et al., 2003; Kantheti et al., 1998). This indicates that unknown tissue-specific and brain-region-specific modifiers affect the function of sorting complexes.

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