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Contribution of AP2 and AP180 to clathrin function in *Dictyostelium discoideum*

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Contribution of AP2 and AP180 to clathrin function in *Dictyostelium discoideum*

by

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Dedication

To my husband, my parents and my daughter

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Contribution of AP2 and AP180 to clathrin function in *Dictyostelium discoideum*

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AP2 complex protein is an essential clathrin adaptor protein during clathrin mediated endocytosis. However, this view has been challenged in simple organisms. To gain insight into this conflict, the role of AP2 in clathrin localization and other clathrin related processes were assessed in *Dictyostelium discoideum*. In *Dictyostelium*, deleting function AP2 caused mild phenotypes in clathrin membrane localization, cytokinesis, osmoregulation and cell development. This supported the idea that AP2 have significant roles in multicellular organisms but not in unicellular system.

Clathrin mediated processes carries important function not only on the plasma membrane but also on some internal organelles. But clathrin coated vesicles on internal organelles are not as well studied as on the plasma membrane. To understand more of the clathrin coated vesicles on internal organelles, the clathrin coated vesicles on *Dictyostelium discoideum* contractile vacuole were studied. Contractile vacuole associated clathrin coated vesicles contained clathrin adaptor proteins AP2, AP180, and

epsin but not Hip1r. The absence of AP180 or AP2 produced abnormal large vacuoles, but the absence of epsin did not cause any detectable contractile vacuole abnormality. The enlarged contractile vacuoles in AP180 minus cells were caused by excessive homotypic fusion among contractile vacuoles. Using both GST-pull down and immunostaining AP180 was identified as the possible adaptor protein for a contractile vacuole-associated SNARE protein, Vamp7B. Therefore recycling Vamp7B from contractile vacuole by AP180 through clathrin coated vesicles could be an efficient way to prevent excessive homotypic fusions among contractile vacuoles. *Dictyostelium* contractile vacuoles offer a valuable system to study clathrin coated vesicles on cell internal organelles.

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Chapter 1: Introduction

1.1 THE CLINICAL SIGNIFICANCE OF CLATHRIN MEDIATED ENDOCYTOSIS

Clathrin mediated endocytosis is the major pathway for eukaryotic cells to internalize important hormones and nutrients, such as EGF (epidermal growth factor) and LDL (low density lipoprotein) through clathrin coated vesicles. This process involves many proteins, membranes and the actin cytoskeleton (Brodsky *et al.*, 2001; Smythe and Ayscough, 2006). The formation of clathrin coated vesicles during endocytosis occurs when clathrin adaptor proteins recognize specific transmembrane receptors on the cytosolic surface of the plasma membrane. After vesicles bud and pinch off from the plasma membrane, internalized materials are then transported to either endosomes, where the vesicle contents are either sent lysosomes or recycled back to the cell surface (Mousavi *et al.*, 2004).

Clathrin and other clathrin coated components have been implicated in various human diseases. It is well known that the LDL (low density lipoprotein) receptor is internalized into cells in clathrin coated vesicles to remove LDL from the blood stream. Defects in LDL receptor internalization can cause hypercholesterolemia, a condition that can lead to atherosclerosis (Anderson *et al.*, 1977). In Alzheimer's disease patients' the expression level of the clathrin adaptor protein AP2 decreases in the frontal cortex of the brain (Yao *et al.*, 2000). In addition to AP2, the clathrin assembly protein AP180 is linked to human diseases: AP180 punctae are reduced in the superior frontal gyrus in the brains of Alzheimer's disease patients while the AP180 non-neuron homologue CALM has been identified as a lymphoid myeloid leukemia gene (Dreyling *et al.*, 1996; Yao *et al.*, 1999). The expression level of synaptojanin, a molecule that plays roles in clathrin-mediated synaptic vesicle endocytosis, is significantly elevated in the cerebral cortex of

patients with Down syndrome (Arai *et al.*, 2002). In multiple recent studies, clathrin assembly protein epsin 4 has been demonstrated to be significantly associated with schizophrenia (Liou *et al.*, 2006; Tang *et al.*, 2006). Another clathrin coated vesicle component, Hip1r, is also linked to Huntington's disease and bipolar disorder (Provencal *et al.*, 2004; Legendre-Guillemin *et al.*, 2005). In addition, there are multiple lines of evidence showing that clathrin mediated endocytosis is also involved in the internalization of influenza and hepatitis C viruses into cells (DeTulleo and Kirchhausen, 1998; Blanchard *et al.*, 2006).

1.2 THREE LAYERS OF CLATHRIN COATED VESICLES ON THE PLASMA MEMBRANE

1.2.1 Clathrin coat---the outer layer

Since clathrin was first identified in 1970s by Barbara Pearse using pig brain extracts, clathrin coats have been well studied (Pearse, 1975). Studies showed that clathrin is a heterodimer of clathrin heavy chain (~190KDa) and clathrin light chain (~25KDa) (Kirchhausen *et al.*, 1983; Ungewickell, 1983). Three clathrin heavy chains with their own associated clathrin light chains trimerize to form triskelia which serve as building blocks from which the clathrin polyhedral lattices are formed (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981; Kirchhausen, 2000; Edeling *et al.*, 2006). These clathrin lattices make up the outer layer of the clathrin coated vesicles (Figure 1.1A).

The amino acid sequence of clathrin heavy chain is highly conserved from yeasts to mammals. Crystallography revealed that clathrin heavy chain has a globular N-terminal terminal domain, a curved region, a linker, a proximal helical leg and a C-terminal end (Ungewickell, 1999). C-terminal domains of three clathrin heavy chain

protein molecules join together to form the hub of a triskelion. Heavy chain proximal leg domains then intertwine with adjacent triskelia to form a clathrin lattice (Fotin *et al.*, 2004). The N-terminal domain forms a seven-bladed β-propeller which contains binding sites for various endocytic proteins, including the AP2 complex. Most of the clathrin interaction proteins bind clathrin heavy chain through their clathrin binding box (ter Haar *et al.*, 1998) (Figure1.1B).

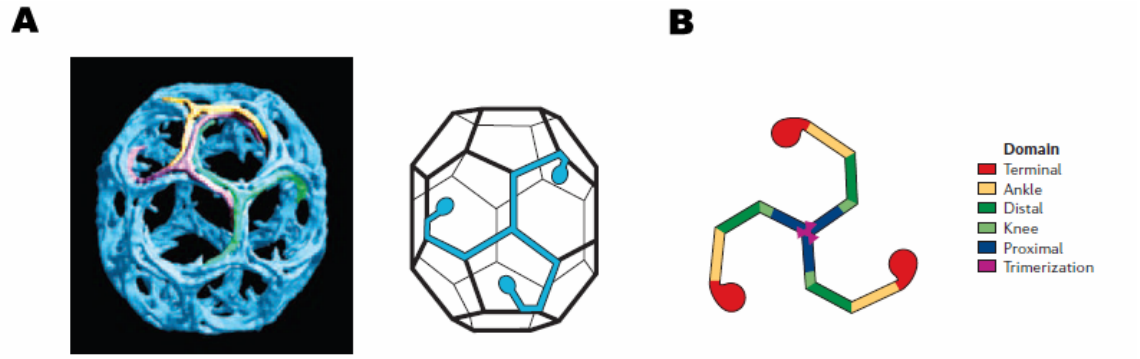


Figure 1.1 Clathrin (A). Clathrin-cage reconstructions. In the left panel, three clathrin triskelia, pink, yellow and green, intertwine with each other. In the right panel, a clathrin barrel with a single triskelion is highlighted in blue. (B). A clathrin triskelion, various domains are highlighted using different colors.
(Both Figure 1.1 A and B are adapted from Edeling *et al.*, 2006)

Mammalian cells have two clathrin light chains homologues, each of which is encoded by an independent gene (Jackson *et al.*, 1987; Kirchhausen *et al.*, 1987; Jackson and Parham, 1988). An acidic motif at the N-terminus of the light chain binds to the proximal legs of clathrin heavy chain and the C-terminus lies adjacent to the vertex of the heavy chain triskelion (Kirchhausen *et al.*, 1983; Ungewickell, 1983; Liu *et al.*, 1995).

Despite being much smaller than the heavy chain, clathrin light chain contributes to the regulation of triskelion assembly. Clathrin assembly occurs spontaneously *in vitro* at low pH and is mediated by clathrin assembly proteins *in vivo* at physiological pH. Clathrin light chains can inhibit clathrin heavy chains assembly through inhibiting high affinity salt bridge formation at physiological pH. This allows clathrin coat formation to be accurately regulated by additional cellular factors (Ybe *et al.*, 1998). *In vivo* studies in budding yeast and *Dictyostelium* showed that missing functional clathrin light chain compromises clathrin function and causes some mutant phenotypes, suggesting an important contribution of the light chain to clathrin functions (Huang *et al.*, 1997; Wang *et al.*, 2003).

1.2.2 Clathrin adaptor proteins ---the middle layer

In clathrin coated vesicles, many clathrin related proteins, including clathrin adaptor proteins and other accessory proteins, build the middle layer of clathrin coated vesicles, connecting the outer layer clathrin coat and the inner layer transmembrane cargo. Clathrin adaptor proteins are required to recognize transmembrane sorting signals, assemble clathrin cages, catalyze membrane curvature and to recruit other important proteins to facilitate the internalization of clathrin coated vesicles. The following are clathrin adaptor proteins that were studied in my Ph.D project.

1.2.2.1 AP2

The tetrameric adaptor protein complex AP2 is the second most abundant component, after clathrin, in clathrin coated vesicles. AP2 binds clathrin, plasma membrane phospholipids, sorting signals and other accessory proteins (Owen *et al.*,

2004). It is thought that clathrin mediated endocytosis is triggered by the recruitment of AP2 onto the plasma membrane from cytosol. AP2 then binds the sorting signals of transmembrane cargo and recruits clathrin onto the membrane. After clathrin coated vesicles bud off from the plasma membrane, clathrin and AP2 dissociate from the vesicles (Conner and Schmid, 2003).

The AP2 complex consists of two large subunits, α and β2 (~100kDa each), a medium subunit, μ2 (~50kDa), and a small subunit, δ 2 (~16kDa) (Figure1.2). The α and β2 subunits each contain an N-terminal trunk domain and a globular C-terminal appendage domain connected by a flexible hinge domain. Both AP2α and β2 appendage domains recruit clathrin accessory proteins, while the β2 subunit also interacts with clathrin and promotes clathrin assembly (Collins *et al.*, 2002; Kirchhausen, 2002; Mousavi *et al.*, 2004). The μ2 subunit is primarily responsible for cargo binding: its C-terminus binds to tyrosine-based sorting signals in transferrin receptor, LDL receptor and EGF receptor (Ohno *et al.*, 1995). There are two plasma membrane PtdIns-4,5-P2 binding sites on the AP2 complex. The interaction between the N-terminal domain of the α subunit and PtdIns-4,5-P2 is the initial and essential step, while the second step, interaction of μ2 and PtdIns-4,5-P2, stabilizes the AP2/plasma membrane association (Honing *et al.*, 2005).

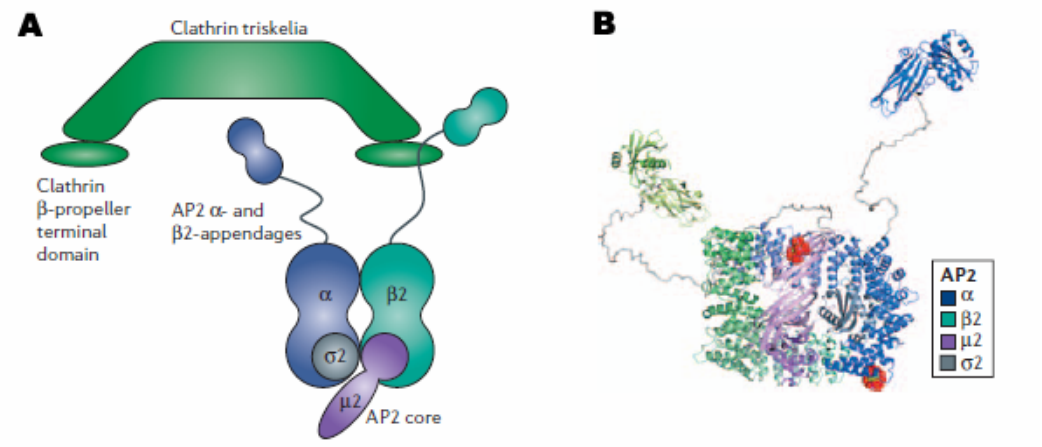


Figure 1.2 AP2 (A). Tetrameric AP2 complex has four subunits. α, β2, μ2 and sigma 2. AP2 binds to clathrin throught it β2 hinge domain. (B). Models of AP2 were are revealed by X-ray crystallography. (Both Figure 1.1 A and B are adapted from Edeling et al., 2006)

1.2.2.2 AP180

In addition to AP2, the clathrin adaptor protein AP180 can also assemble clathrin lattice (Ford *et al.*, 2001). This makes AP180 another important player in clathrin mediated endocytosis. AP180/CALM is implicated in the efficient assembly of uniformly sized clathrin cages (Ahle and Ungewickell, 1986; Heuser *et al.*, 1987; Prasad and Lippoldt, 1988; Ye and Lafer, 1995; Zhang *et al.*, 1998; Ford *et al.*, 2002). A conserved N-terminal ANTH (AP180 N-terminal homology) domain recruits AP180/CALM to PtdIns(4,5)P_2 at the plasma membrane (Norris *et al.*, 1995; Ye *et al.*, 1995; Ford *et al.*, 2001; Mao *et al.*, 2001). AP180/CALM binds directly to clathrin through its conserved clathrin motifs, DLL and/or L(L,I)(D,E,N)(L,F)(D,E) (Morgan *et al.*, 2000; ter Haar *et al.*, 2000). It also contains DPW/DPF motifs which bind both α and β2 subunits of AP2 (Owen *et al.*, 1999; Owen *et al.*, 2000).

Direct binding between AP2 and AP180 has been shown *in vitro* and accounts for their synergistic clathrin assembly activity. AP180 alone assembles clathrin monomers into lattices about four times better than AP2 alone, but when combined with AP2, their assembly activity is greater than the simple adding of assembly ability of each protein (Lindner and Ungewickell, 1992; Hao *et al.*, 1999). Immunofluorescence images also show colocalization of the two proteins within punctae (Hinrichsen *et al.*, 2003; Meyerholz *et al.*, 2005). However a reduction of AP2 in HeLa cells only moderately reduces the membrane associated AP180/CALM, suggesting that the interaction between AP2 and AP180 is not critical for AP180/CALM function (Hinrichsen *et al.*, 2003). AP180/CALM is also implicated in cargo internalization: knocking down AP180/CALM in HeLa cells affects the internalization of EGF receptors but not transferrin receptor (Huang *et al.*, 2004). This finding also puts AP180/CALM as one possible candidate that can recognize and internalize some clathrin mediated endocytosis cargo.

1.2.2.3 Epsin

The epsin ENTH (Epsin N-terminal Homology) domain is highly related to the AP180 ANTH domain (Ford *et al.*, 2002). Similarly, the ENTH domain binds specifically to PtdIns (4, 5) P_2 at the plasma membrane and promotes clathrin assembly. Unlike AP180, which can only assemble a flat clathrin lattice, part of the epsin ENTH domain inserts into the plasma membrane bilayer and induces membrane curvature and clathrin coated pit invagination *in vitro* (Itoh *et al.*, 2001; Ford *et al.*, 2002). At the carboxyl-terminus, epsin binds clathrin and AP2 through similar motifs as AP180 (Dell'Angelica, 2001). This has also been shown in immunofluorescence images in which epsin colocalizes with both clathrin and AP2. In addition, epsin punctae are reduced in AP2 μ2 depleted cells (Hinrichsen *et al.*, 2003; Motley *et al.*, 2003). It has

been suggested that epsin could interact with some ubiquintinated cargo such as EGF receptors through its Ubiquitin Interacting Motifs (UIMs) (Nakashima *et al.*, 1999; Hofmann and Falquet, 2001; Polo *et al.*, 2002; Aguilar *et al.*, 2003; Barriere *et al.*, 2006). In addition, in *Drosophila* and *C. elegans*, epsin is also important for the Notch/Delta pathway (Overstreet *et al*., 2003; Overstreet *et al*., 2004; Tian *et al*., 2004). Only after epsin-dependent internalization is the Delta ligand able to activate Notch (Wang and Struhl, 2004; Wang and Struhl, 2005). All these data suggest that, like AP2, epsin could also help select and internalize cargo from the plasma membrane.

1.2.2.3 The Sla2/Hip1 family

Sla2/Hip1 family members include yeast Sla2p, mammalian Hip1 and Hip1r. These family members also contain an N-terminal ANTH domain which interacts with PI (4, 5) P2 in the plasma membrane (Itoh *et al.*, 2001; De Camilli *et al.*, 2002; Legendre-Guillemin *et al.*, 2004). Adjacent to the ANTH domain Sla2/Hip1 has a coiled-coil domain which binds to clathrin (Engqvist-Goldstein *et al.*, 1999; Mishra *et al.*, 2001; Henry *et al.*, 2002). At the C-terminus, Sla/Hip1 has a THATCH (Talin-Hip1/R/Sla2p Actin-Tethering C-Terminal Homology) domain which binds to F-actin (McCann and Craig, 1997; Yang *et al.*, 1999; Brett *et al.*, 2006).

Yeast Sla2p mutants are deficient in endocytosis and the central coiled-coil domain binds to clathrin light chain (Raths *et al.*, 1993; Henry *et al.*, 2002). The Sla2p THATCH domain interacts with yeast epsin which suggested a close relationship between epsin and Sla2p/Hip1 family members (Baggett *et al.*, 2003). Hip1 (Huntingtin Interacting Protein 1), mostly expressed in neurons, binds to AP2 and clathrin light chain through binding motifs and can also promote clathrin assembly (Kalchman *et al.*, 1997; Wanker *et al.*, 1997; Legendre-Guillemin *et al.*, 2002). Hip1r, the Hip1 related protein,

colocalizes with both AP2 and clathrin (Engqvist-Goldstein *et al.*, 1999; Engqvist-Goldstein *et al.*, 2001). Strong evidence has shown that Hip1r can link actin filaments to the clathrin coat (Chen and Brodsky, 2005). Therefore, Hip1r offers a very important tool to study the actin dynamics involved in clathrin mediated trafficking.

1.3 ACTIN IN CLATHRIN MEDIATED TRAFFICKING

The dynamic polymerization of the actin cytoskeleton has direct roles during the formation of clathrin coated vesicles. In both mammalian cells and *S.cerevisiae*, inhibiting actin filament assembly blocks endocytosis (Ayscough, 2000; Fujimoto *et al.*, 2000). Further experiments demonstrated that clathrin coated vesicle invagination in mammalian cells can initiate without actin, but efficient vesicle scission requires functional actin (Yarar *et al.*, 2005). In contrast, *S.cerevisiae* cells need actin for both vesicle invagination and scission (Kaksonen *et al.*, 2003). In addition, the localization of actin also suggests an important function of actin in endocytosis. Immunostaining in *S.cerevisiae* cells showed a colocalization between actin patches and many endocytic proteins (Engqvist-Goldstein and Drubin, 2003). Moreover, electron micrographs from mammalian cells also revealed a possible association between clathrin coated vesicles and action filaments (Salisbury *et al.*, 1980). Finally, actin filaments were also found at the sites of clathrin mediated endocytosis in neuronal synapses, further suggesting an important role for actin for clathrin function (Shupliakov *et al.*, 2002).

Actin has the ability to generate mechanical forces through two different mechanisms, one of which is directly driven by F-actin polymerization. This kind of polymerization-driven force is responsible for the motility of certain pathogens inside of their host cells, such as *Listeria* and *Shigella* (Frischknecht and Way, 2001; Gouin *et al.*, 2005). It has also been proposed that during endocytosis, the nucleation of actin

filaments is activated at the edges of the invagination pits. These continuously growing actin filaments then bind to the endocytic coat and form an F-actin network that drags endocytic coated pits into invagination (Mulholland *et al.*, 1994; Rodal *et al.*, 2005). In support of this theory, recent studies have identified several proteins that link the clathrin coat and F-actin, such as Sla2/Hip1r family and Pan1/Eps15 (Engqvist-Goldstein *et al.*, 1999; Kaksonen *et al.*, 2003).

The second mechanism of generation of actin forces is through motor proteins, such as myosin VI. Evidence suggests that myosin VI colocalizes with clathrin pits and is able to pull endocytic vesicles away from the plasma membrane (Buss *et al.*, 2001a; Buss *et al.*, 2001b; Spudich *et al.*, 2007). Thus myosin VI may also facilitate clathrin coated vesicle budding.

1.4 LIFE CYCLE OF CLATHRIN COATED VESICLES

The formation of clathrin coated vesicles is initiated at specific and limited assembly spots on the plasma membrane (Santini *et al.*, 2002). The life cycle of clathrin coated vesicles during endocytosis is a dynamic process which involves many proteins, plasma membrane and actin filaments. Despite the variations among different organisms, the basic steps of the life cycle of the vesicles are very similar: clathrin coat assembly, clathrin coated pits invagination, scission and uncoating.

1.4.1 Clathrin coat assembly

The assembly of clathrin triskelia into clathrin lattice is the first step of clathrin mediated endocytosis. Although clathrin triskelia have some self assembly ability, clathrin assembly proteins are required to regulate this process (Liu *et al.*, 1995; Brodsky

et al., 2001). Adaptor protein complex AP2 is believed to be able to specify the sites of clathrin assembly (Brodsky, 1988). AP2 binds to plasma membrane through its two plasma membrane phosphoinositide binding sites, one on α subunit and one on μ2 subunit. In addition, AP2 also binds to the internalization signals on the transmembrane receptors, the cargo of the clathrin coated vesicles that will ultimately be internalized. Together, the binding of phosphoinositides and transmembrane receptors recruit AP2 onto the plasma membrane and also determine the initiation sites for clathrin assembly (Pearse and Crowther, 1987; Iacopetta *et al.*, 1988; Zhang *et al.*, 1994; West *et al.*, 1997; Jost *et al.*, 1998; Gaidarov and Keen, 1999).

After AP2 specifies the location of clathrin assembly on the plasma membrane, the AP2 β2 subunit hinge domain binds clathrin heavy chain and promotes clathrin lattice polymerization (Shih *et al.*, 1995; Gaidarov and Keen, 1999). However, AP2's central role in clathrin assembly nucleation has been challenged. Depletion of AP2 complexes from *S.cerevisiae* by genetic knockout does not abolish clathrin coated vesicles (Huang *et al.*, 1999; Yeung *et al.*, 1999). So it is now believed that, at least in some organisms, clathrin assembly on the plasma membrane could be AP2 independent.

Other than AP2, AP180/CALM is also an important player on the assembly of plasma membrane clathrin. AP180/CALM could be acting as a regulator of vesicle size (Ahle and Ungewickell, 1986; Heuser *et al.*, 1987; Prasad and Lippoldt, 1988; Ye and Lafer, 1995; Zhang *et al.*, 1998). In addition, having the ability to promote clathrin assembly, AP180 binds to AP2 such that the resulting AP180-AP2 complexes assemble clathrin lattice with higher efficiency than the simple addition of each protein (Lindner and Ungewickell, 1992).

1.4.2 Clathrin coated pit invagination

There are two steps of clathrin coated pit invagination: the early formation of shallow coated pits and the later formation of deeply invaginated pits. These two steps both require certain degrees of membrane deformation (McMahon and Gallop, 2005).

There are three important factors involved during the formation of shallow coated pits on the plasma membrane. One major factor is clathrin coat assembly which has been suggested to provide the driving force for membrane invagination (Moore *et al.*, 1987; Mahaffey *et al.*, 1989; Jin and Nossal, 1993; Hinrichsen *et al.*, 2006). However, others have argued that forces other than clathrin also drive the invagination. One of these forces is the asymmetry of two plasma membrane leaflets. Being asymmetric, the two layers of the plasma membrane could respond differently to various perturbations. This differential response can cause one leaflet to expand more than the other and therefore induce membrane curvature. For example, the insertion of epsin ENTH domain can cause membrane curvature under this mechanism. Epsin has showed to be able to tubulate liposomes and promote the formation of clathrin coated pits on monolayer membranes. The epsin ENTH domain helix 0 inserts into the inner leaflet of the plasma membrane bilayer causing an increase in the surface of the leaflet, thus inducing membrane bending (Ford *et al.*, 2002).

The formation of deep clathrin coated pits involves a different set of proteins such as endophilin and dynamin. When endophilin function was interrupted in lamprey synaptic vesicle invagination stopped at a shallow stage (Ringstad *et al.*, 1999). In addition, dynamin rescued the formation of deeply invaginated clathrin coated pits inhibited by overexpression of the SH3 domain of amphiphysin, a linker protein of dynamin and clathrin coats. This suggests that the requirement of dynamin is in the later stage of vesicle budding (Hill *et al.*, 2001).

After full invagination from the plasma membrane, clathrin coated vesicles get ready to detach from the membrane.

1.4.3 Scission--Detachment of clathrin coated vesicles

The neck of a deeply invaginated vesicle specifies the site of vesicle scission. This vesicle neck must be constricted sufficiently to release the vesicle from the plasma membrane. During vesicle scission, dynamin, a GTPase, is recruited to the clathrin coated pits by amphiphysin. Dynamin induces its own GTP hydrolysis and triggers conformational changes that provide mechanical forces around the neck of the invaginated pits to complete vesicle scission (Baba *et al.*, 1995; Hinshaw and Schmid, 1995). In addition to GTP hydrolysis, dynamin may also recruit and activate other effectors, such as endophilin which can drive vesicle scission through its lipid transferase activity (Kozlov, 2001). Dynamin also interacts with many actin binding proteins including Arp2/3, WASP, profilin, Abp1. Thus, it has been proposed that dynamin is important for vesicle scission because it regulates actin filament polymerization which provides the ultimate mechanic forces to the scission of clathrin coated vesicles (Witke *et al.*, 1998; McNiven *et al.*, 2000; Kessels *et al.*, 2001).

1.4.4 Uncoating

After clathrin coated vesicles are released from the plasma membrane, clathrin lattices are disassembled while the vesicles are being transported to next organelle. During this uncoating process, Hsc70 and auxillin are targeted onto clathrin coated vesicles and then drive ATP hydrolysis. This ATP hydrolysis provides the energy required for clathrin coat disassembly. At the same time, phosphorylated AP2 β2

subunit also prevents the adaptor complex from binding clathrin once AP2 leaves the vesicle (Schlossman *et al.*, 1984; Ungewickell *et al.*, 1995; Umeda *et al.*, 2000).

1.5 CLATHRIN ON INTERNAL ORGANELLES

Clathrin coated vesicles are not limited to the plasma membrane. Immunoflurescence images have localized clathrin onto the *trans*-Golgi network (TGN) and endosomes.

At the TGN, clathrin coated vesicle deliver newly synthesized lysosomal hydrolases to the endosomal compartments (Friend and Farquhar, 1967; von Figura and Weber, 1978; Gonzalez-Noriega *et al.*, 1980). The life cycle at the TGN is similar to that of the plasma membrane with some different proteins involved. The differences are highlighted as following.

Clathrin coat assembly at the *trans*-Golgi network is activated by the recruitment of ARF1, a GTPase to the membrane. When activated by binding GTP, ARF1 recruits clathrin adaptor proteins AP1 and GGAs, a class of monomeric clathrin adaptor proteins to the TGN (Stamnes and Rothman, 1993; Seaman *et al.*, 1996; Donaldson *et al.*, 2005). The monomeric GGAs and the tetrameric AP family member AP1 serve as adaptor proteins at the TGN and they bind the sorting signal of trans-Golgi membrane receptors. GGAs bind to Asp-X-X-Leu-Leu motifs whereas AP1 binds with (DE)XXXL(LI) motifs (Austin *et al.*, 2002; Crottet *et al.*, 2002; Shiba *et al.*, 2002). In addition AP1 interacts with phosphatidylinositol-4-monophosphate (PI4P) and assembles clathrin into lattice on TGN membranes (Doray and Kornfeld, 2001; Heldwein *et al.*, 2004). GGAs can also assembly clathrin into lattice, but a recent study done with GGA1 showed that 10% of the clathrin structure it assembles is in the form of clathrin tubules, suggesting a unique assembly ability for GGAs (Zhang *et al.*, 2007). After been recruited to the TGN, AP1

and GGAs also recruit a complex of clathrin accessory/regulatory proteins that are unique for TGN clathrin coated vesicle formation, including epsinR, Eps15 (Kent *et al.*, 2002; Hirst *et al.*, 2003; Lui *et al.*, 2003). However, the later stages of the clathrin coated vesicles budding and scission at TGN involve similar proteins as on the plasma membrane, including Hip1r, dynamin, endophilin and actin cytoskeleton (reviewed by (McNiven and Thompson, 2006)).

Other than the plasma membrane and the TGN localization, clathrin coated vesicles are also localized on endosomes in mammalian cells with AP1 being the only known endosomal adaptor. First observed in 1996, clathrin coated vesicles associated with endosomes are smaller than those on the plasma membrane (Stoorvogel *et al.*, 1996). AP1 was localized onto endosome when retrograde transport between the Golgi and endosomes was blocked by BFA, suggesting a role for AP1 on the endosomes (Mallard *et al.*, 1998; Hinners and Tooze, 2003). At the endosome, clathrin coated vesicles are involved in sorting Shiga toxin from late endosomes to the TGN (Lauvrak *et al.*, 2004). However, a different study also suggests that endosome associated clathrin coated pits are responsible for the recycling of the transferrin receptors back to the plasma membrane (van Dam and Stoorvogel, 2002). It was suggested that AP3 may play a role on the endosomes as well. But whether AP3 is related to clathrin function is still under debate (reviewed by (Robinson and Bonifacino, 2001)).

Recent studies in tobacco cells, revealed that clathrin localized to another internal cell organelle, the phragmoplast during the late stage of cell division (late anaphase and telophase) (Tahara *et al.*, 2007). The phragmoplast is a plant-cell-specific organelle that serves as a scaffold for cell plate assembly and formation of a new cell wall separating the two daughter cells during late cytokinesis. The localization of clathrin on the phragmoplast suggests a possible role for clathrin in the organization of the phragmoplast

during cytokinesis in tobacco cells. However, how clathrin functions and which clathrin adaptor proteins participate at this cell stage remain undetermined.

1.6 CLATHRIN AND ITS PARTNERS IN *DICTYOSTELIUM DISCOIDEUM*

The slime mode *Dictyostelium discoideum* is a social amoeba with six chromosomes and a 34Mb genome (Cox *et al.*, 1990; Loomis *et al.*, 1995). In the natural world, *Dictyostelium* cells exist as two phases: a vegetative phase and a developmental phase. Upon starvation, the vegetative *Dictyostelium* cells enter a developmental program in which about 100,000 amoebae cells aggregate, differentiate and then form a multicellular fruiting body consisting of a sorus full of spores on the top of a stalk. Aggregation is mediated by chemotaxis to cAMP produced by starving cells (Kessin, 2001). *Dictyostelium discoideum* is a unique model system with powerful molecular genetic tools for studying some fundamental cellular pathways. First, many of *Dictyostelium* genes show a high degree of sequence similarity to genes in vertebrate species. Secondly, relative to yeast, *Dictyostelium* cells are very accessible for fluorescence and electron microscopy because of their large size (~10 micron) and lack of a cell wall. Third, *Dictyostelium* is a haploid system in its vegetative state and the entire genome has already been sequenced which makes genes disruption very manageable. Finally, it is relatively inexpensive and easy to grow *Dictyostelium* in the large quantities necessary for biochemical analysis.

Previous studies of clathrin mediated processes in *Dictyostelium discoideum* revealed that cells clathrin-coated pits are associated with the plasma membrane and clathrin is important for pinocytosis, cytokinesis, osmoregulation and development (O'Halloran and Anderson, 1992b; Niswonger and O'Halloran, 1997b, 1997a; Gerald *et al.*, 2001; Wang *et al.*, 2006).

Furthermore, various clathrin related endocytic proteins have been cloned and studied in *Dictyostelium*, including AP1 γ and μ1 subunit, AP180, epsin, and Hip1r. AP1 in *Dictyostelium* colocalizes with clathrin on the TGN and is required for the biogenesis of contractile vacuole, an osmoregulatary system. Deleting AP1 also causes a delay in the developmental cycle in *Dictyostelium* (Lefkir *et al.*, 2003). *Dictyostelium* AP180 colocalizes with clathrin at the plasma membrane and at the contractile vacuole and is also involved in contractile vacuole function. AP180 depleted cells have enlarged contractile vacuoles and are osmosensitive (Stavrou and O'Halloran, 2006). Two other clathrin accessory proteins, epsin and Hip1r, also colocalize with clathrin on the plasma membrane. However, unlike other clathrin adaptor proteins, epsin and Hip1r are involved in *Dictyostelium* spore formation. Cells missing epsin or Hip1r produce round and fragile spores instead of the ovoid and robust spores from wild type cells (Repass *et al.*, 2007; Brady *et al.*, 2008).

1.7 DICTYOSTELIUM CONTRACTILE VACUOLES

1.7.1 Overview of the contractile vacuole system in *Dictyostelium discoideum*

Living in soil, the protozoa *Dictyostelium* has a well characterized osmoregulatory organelle called the contractile vacuole complex which collects and expels excess water from inside of the cell. The contractile vacuole (CV) complex is a highly dynamic organelle consisting of bladders (cisternae) connected by a network of tubules. The contractile vacuole tubules and bladders in *Dictyostelium* cells are interconvertable, and the numbers of bladders and tubules also vary as needed (Gerisch *et al.*, 2002) (Figure 1.3). At the beginning of the contractile vacuole life cycle, the expansion phase, water is collected into lenticular shaped bladders via proton pumps (v-H+ATPase). V-ATPase

is found throughout the whole contractile vacuole system membrane and is believed to drive the water exchange between the cell cytosol and contractile vacuole by creating a positive charge inside of the contractile vacuole. This electrical charge can cause anions

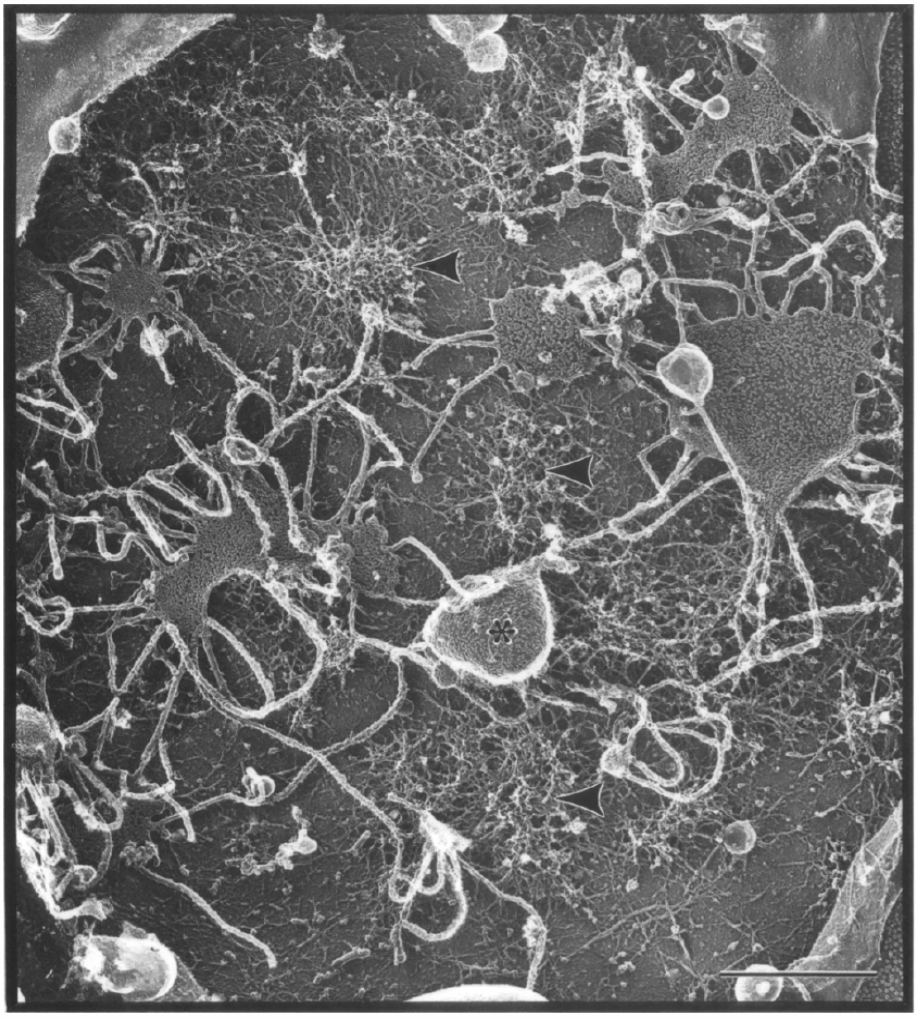


Figure 1.3 EM picture of interior of a *Dictyostelium* amoeba showing an extensive, interconnected contractile vacuole system. One swollen cisterna being partially filled at the moment of cell fixation are indicated with asterisk. Visible cytoskeletal structures are also showed with arrowheads. Bar, 1μm (Heuser et al., 1993).

and water to flow into the contractile vacuole following their concentration gradients (Heuser *et al.*, 1993). At the end of this expansion stage, the lenticular bladder rounds up to form a vacuole-like compartment. Soon after the bladder reaches its maximum size, it moves toward the cell surface before it fuses with the plasma membrane forming a temporary pore and discharging its contents into the extracellular space (Figure 1.4 A and B). During expulsion, the bladder membrane folds back into tubules (Heuser *et al.*, 1993; Gerisch *et al.*, 2002). Despite the brief contact between contractile vacuole membrane and the plasma membrane, evidence has shown that these two membranes actually never mix. A contractile vacuole membrane marker (GFP-Dajumin) does not mix with the plasma membrane after contractile vacuole discharge (Gabriel *et al.*, 1999). In addition, in contrast to the plasma membrane, the contractile vacuole membrane is full of proton pumps and devoid of actin filaments and myosin (Clarke *et al.*, 2002; Gerisch *et al.*, 2002; Heuser, 2006).

Contractile vacuoles are known to accumulate and expel water for the cell, but the internal contents of contractile vacuoles are not fully understood. One study done in the amoeba *C.carolinensis* showed that contractile vacuoles contain a very high concentration of Na+ and a very low concentration of K^+ compared to the cytosol (20mM Na^+ and 4.6mM K^+ in contractile vacuole compared to the 0.6mM of Na^+ and 31mM of K^+ in cytosol) (Riddick, 1968). Heuser *et al* further proposed that in order to maintain the ion balance inside of cells when contractile vacuole continuously release Na^+ and K+, the cells may exchange the byproduct of respiration and nitrogen metabolism (Heuser *et al.*, 1993).

Some studies have also proposed a role for the contractile vacuole in the regulation of intracellular Ca^{2+} concentration. For example, the Ca^{2+} binding protein calmodulin has been localized onto contractile vacuoles. Furthermore, it has been

shown that purified contractile vacuoles can store and release Ca^{2+} (Zhu and Clarke, 1992; Malchow *et al.*, 2006).

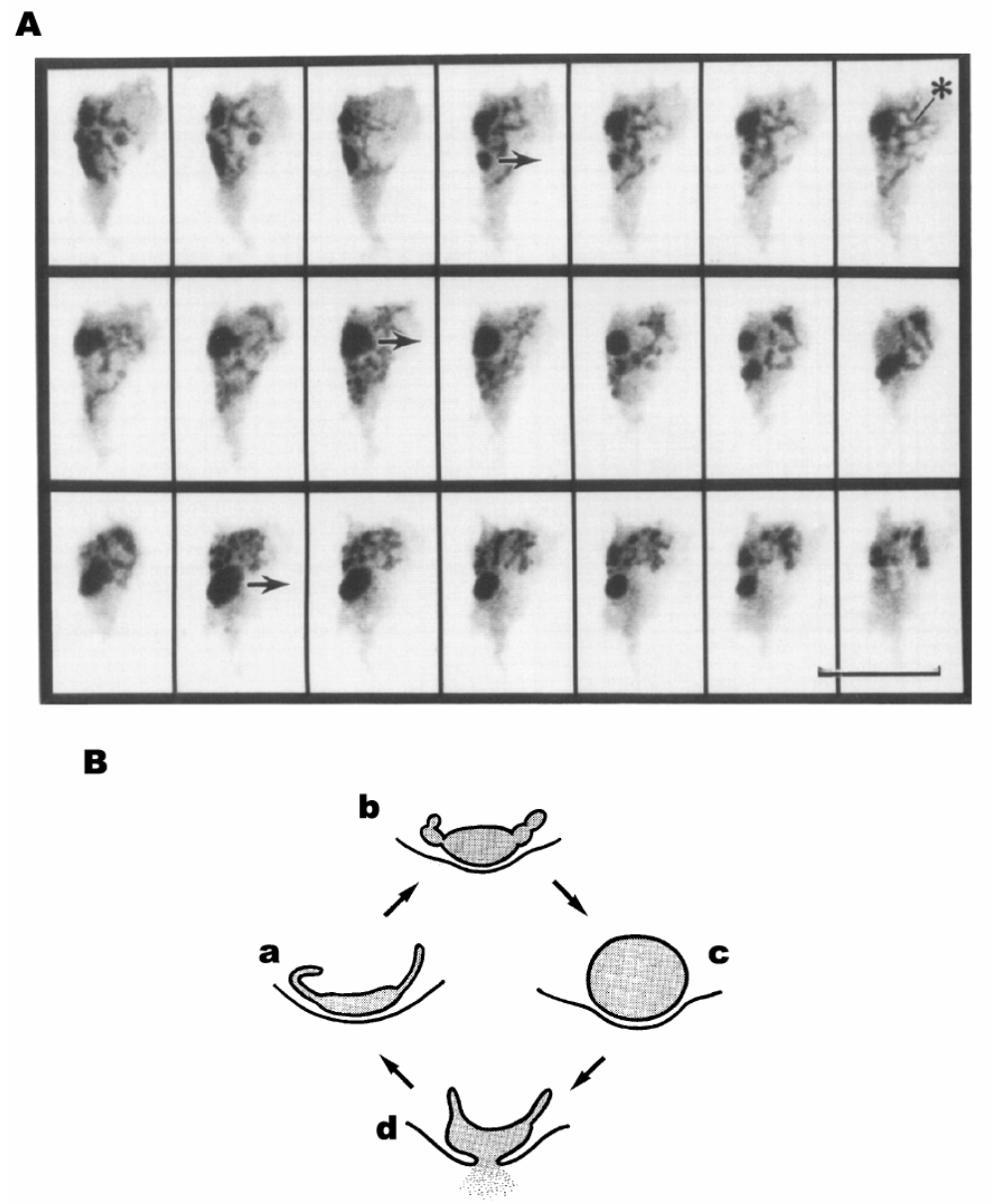


Figure 1.4 Contractile vacuole cycle. (A). Time-lapse video microscopy of a *Dictyostelium* cell for showing the cycle of contractile vacuole from expansion to expel. Recording starts in the upper left panel and proceeds to the lower right (4 seconds intervals). Contractile vacuoles complexes appear dark against the lighter grey of the cell

bottom. Arrows indicate the onsets of three discharge events. A particularly example of a tubulo-cisternal intermediate in this process is indicated at the asterisk. Bar, 10μm. (B). Proposed cycle of contractile vacuole filling and discharge in *Dictyostelium.* In (a) is a swelling of a particular contractile vacuole bladder from water accumulation. The volume of the bladder in (b) increases while the tubules also expanded and shortened. Then the shortened tubules merged into the main bladder and become one round vacuole. This vacuole then moves close to the plasma membrane and opened a temporary pore to dump its content and collapses at the plasma membrane in (d). (Adapted from Heuser *et al*., 1993).

Upon discharging, contractile bladder must contact with the plasma membrane at an appropriate site and this step probably involves SNARE proteins, suggesting a possible role of SNARE protein for normal contractile vacuole functions. In addition, the observation of homotypic fusion among dispersed contractile vacuole parts in mitotic cells suggests a role for SNAREs in this process (Gabriel *et al.*, 1999). Although some SNAREs have been identified, including a Syntaxin 7 homologue and α and gamma-SNAP in *Dictyostelium* cells, the roles of SNARE proteins in contractile vacuoles has not been elucidated (Bogdanovic *et al.*, 2000; Weidenhaupt *et al.*, 2000; Bogdanovic *et al.*, 2002; Bennett *et al.*, 2008).

1.7.2 Contractile vacuole resident proteins

Many proteins have been localized onto the contractile vacuole systems in *Dictyostelium*. Among those, calmodulin, Rab-11 like protein, GFP-fused Dajumin and Rh50 are four proteins that localize specifically on contractile vacuoles. Calmodulin, a calcium binding protein is the first protein identified on the contractile vacuole (Zhu and

Clarke, 1992). *Dictyostelium* Rab-11, a relative of mammalian Rab-11 protein, also specifically localizes to the contractile vacuole (Harris *et al.*, 2001). GFP-fused dajumin provides a very useful fluorescent marker for observing contractile vacuole activities in living *Dictyostelium* cells while dajumin by itself is not a contractile vacuole related protein (Gabriel *et al.*, 1999). Rh50, a protein related to the member of the human Rhesus complex is the specific contractile maker used in this study (Benghezal *et al.*, 2001).

There are also proteins enriched in the contractile vacuoles that are also found in other cellular localization. The proton pump v-ATPase is one of them. Antibodies against v-ATPase subunits and GFP labeled v-ATPase subunits have localized v-ATPase to both contractile vacuoles and endo-lysosomal compartments (Heuser *et al.*, 1993; Clarke *et al.*, 2002). The *Dictyostelium* Rab4-like protein and LvsA, a protein related to the mammalian beige/LYST family are also enriched on the contractile vacuoles in addition to endocytic compartments (Bush *et al.*, 1994; Gerald *et al.*, 2002).

Lipophilic styryl dyes, such as FM4-64, FM1-43, FM2-10 are also used to label contractile vacuoles. These dyes diffuse into contractile vacuole membrane within the first two minutes after they integrate into the plasma membrane.

1.7.3 Clathrin and clathrin adaptor protein in contractile vacuoles

Despite the fact that the contractile vacuole complex shares some proteins with endo-lysosomes, those two systems appear to be physically separate from each other. So it seems a little surprising when studies in the contractile vacuoles revealed some important roles for endocytic protein clathrin and clathrin adaptor proteins in contractile vacuoles. Initial studies with *Dictyostelium* cells suggested a role for clathrin in the biogenesis of contractile vacuole complex. In clathrin heavy chain deficient cells, large

contractile vacuoles were replaced by much smaller dispersed vacuoles. Moreover, when placed in water, clathrin heavy chain mutant cells swelled and rounded up while wild type cells maintained their original shape (O'Halloran and Anderson, 1992b). In support of a role for clathrin function at the contractile vacuole, later studies also showed that clathrin light chain is also required for the normal contractile vacuole function. Clathrin light chain-depleted *Dictyostelium* cells are also osmosensitive and have slightly enlarged contractile vacuoles (Wang *et al.*, 2003). Under certain conditions, such as in cells with latrunculin-disrupted actin filaments, clathrin was observed on the contractile vacuole (Heuser, 2006). Moreover, using GFP tagged clathrin light chain, Starvrou clearly localized clathrin onto both pumping the contractile vacuoles bladder and tubules in *Dictyostelium* (Stavrou and O'Halloran, 2006).

Other than clathrin, two clathrin adaptor proteins, AP1 and AP180, have been also linked to contractile vacuoles in *Dictyostelium* cells. Similar to clathrin heavy chain-deficient cells, AP1 μ1 null cells do not have large contractile vacuole like in wild type cells. On the other hand, the μ1 null cells are osmosensitive and contractile vacuole marker Rh50 is mislocalized into the Golgi area. All these data suggests that in addition to clathrin, AP1 is also required for the biogenesis of contractile vacuoles in *Dictyostelium* (Lefkir *et al.*, 2003).

On the other hand, AP180 null cells have a different contractile vacuole phenotype than AP1 mull cells: AP180 null cell have abnormally enlarged contractile vacuoles and are also osmosensitive. In addition, GFP-tagged AP180 punctae were localized onto both the contractile vacuole bladder and tubule (Stavrou and O'Halloran, 2006).

Despite all the studies that have been done, the composition and functions of clathrin coated vesicles on contractile vacuoles still remains unknown.

1.8 SNARE PROTEINS

In the past two decades, SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins have been identified as key elements during different membrane fusion events. During membrane fusion, SNARE proteins from opposite membranes intertwine into a complex. This led to the old classification of SNARE proteins as v-SNAREs (vesicle membrane SNAREs) or t-SNAREs (target membrane SNAREs) (Sollner *et al.*, 1993). However, this terminology now is considered somehow misleading because of the discovery of homotypic fusion events in which SNARE protein serves both on the "vesicle membrane" and the "target membrane" (McNew *et al.*, 2000). As described below, a newer terminology has emerged that classifies SNARE proteins as either "R-SNAREs" or "Q-SNAREs" based on their amino acid sequence.

1.8.1 SNARE protein structure

Most SNARE protein family members contain a transmembrane domain on the C-terminus, a SNARE motif and an independent folded domain on the N-terminus (Fasshauer, 2003; Hong, 2005; Jahn and Scheller, 2006). During membrane fusion, the SNARE motif mediates SNARE protein complex formation from two opposite membranes. SNARE motifs remain as non-folded domains before they meet their appropriate counterparts. When SNARE proteins from two sides, vesicle membrane and target membrane, combine, the SNARE motifs spontaneously intertwine with each other and form a stable helical core complex (Fasshauer, 2003). Despite the sequence differences among different SNARE motifs, the crystal structures of core complexes are highly conserved. In each core complex, four SNARE motifs, one from the vesicle membrane, three from the target membrane, each contribute an α-helix to intertwine into

a coiled coil structure (Sutton *et al.*, 1998; Antonin *et al.*, 2002). The coiled coil region of the SNARE core complex contains three highly conserved glutamine (Q) residues and one conserved arginine (R) residue. Therefore there is a new classification for SNARE proteins into Q-SNARE's and R-SNARE's according to the distribution of these Q or R residues (Fasshauer *et al.*, 1998).

Unlike the highly conserved SNARE motifs, the N-terminal domains of SNARE proteins are variable. Q-SNAREs proteins each contain three helix bundles which are connected to their SNARE motif through a flexible linker (Misura *et al.*, 2002; Dietrich *et al.*, 2003). On the other hand, the R-SNAREs have conserved profilin-like longin domains and some studies suggested that these longin domains may serve as a SNARE protein recruitment sites (Toonen and Verhage, 2003; Pryor *et al.*, 2008) (Figure 1.5).

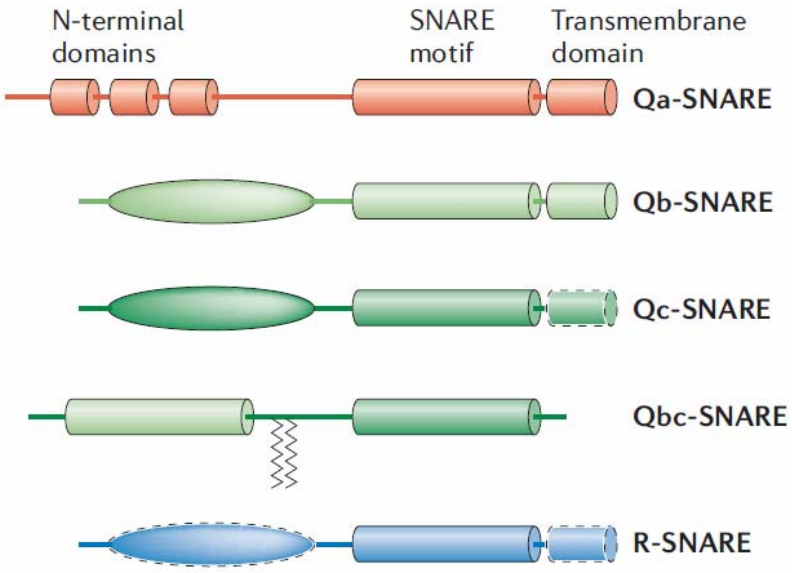


Figure 1.5 The structures of SNAREs. The domain structure of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) subfamilies. Dashed domain borders highlight domains that are missing in some subfamily members. Qa-

SNAREs have N-terminal antiparallel three-helix bundles. The various N-terminal domains of Qb-, Qc- and R-SNAREs are represented by a basic oval shape. Qbc-SNAREs represent a small subfamily of SNAREs (the SNAP-25 subfamily) that contain one Qb-SNARE motif and one Qc-SNARE motif. These motifs are connected by a linker that is frequently palmitoylated (zig-zag lines in the figure), and most of the members of this subfamily function in constitutive or regulated exocytosis. (Jahn, 2006)

1.8.2 SNARE proteins during membrane fusion

It has been suggested that SNARE core complex assembly might be the ultimate driving force for membrane fusion. The association of SNAREs from the two membranes directly initiates fusion. During this step, SNAREs must assemble as a *trans*-complex in which each fusion membrane contributes at least one SNARE domain, and this assembly process starts from the N-terminus SNARE motifs to the C-terminus transmembrane domains (Jahn and Scheller, 2006). As a result of the direct and close contact between the two membranes, and the mechanical force caused by the assembly of SNARE complexes, a fusion pore is formed connecting the outer layers of the two membrane leaflets (Jahn and Grubmuller, 2002) (Figure 1.6). After fusion, all SNAREs of the SNARE complex are on the same fused membranes, resulting in a *cis*-SNARE complex. The disassembly of the *cis*-SNARE complex involves several steps, requiring NSF and the energy provided by ATP-hydrolysis (Sollner *et al.*, 1993). After disassembly, SNARE proteins can be recycled back to be reused.

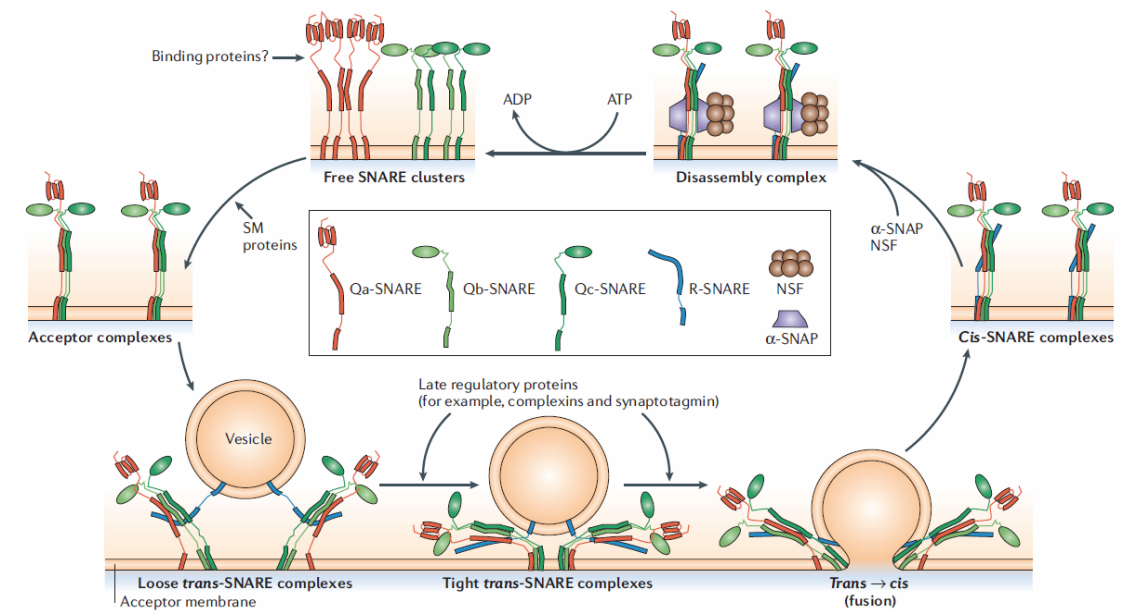


Figure 1.6 The SNARE conformational cycle during vesicle docking and fusion. In this example, three Q-SNAREs on an acceptor membrane and an R-SNARE on a vesicle. Q-SNAREs, which are organized in clusters (top left), assemble into acceptor complexes. Acceptor complexes interact with the vesicular R-SNAREs through the N-terminal end of the SNARE motifs, and this nucleates the formation of a four-helical *trans* complex. *Trans*-complexes proceed from a loose state (in which only the N-terminal portion of the SNARE motifs are 'zipped up') to a tight state (in which the zippering process is mostly completed), and this is followed by the opening of the fusion pore. During fusion, the strained *trans*-complex relaxes into a *cis*-configuration. *Cis*-complexes are disassembled by NSF (*N*-ethylmaleimide-sensitive factor) together with SNAPs (soluble NSF attachment proteins). The R- and Q-SNAREs are then separated by sorting (Jahn,2006).

1.8.3 Trafficking of SNARE proteins

To ensure proper fusion, SNARE proteins are constantly sorted to their initial target membranes. In addition, some SNARE proteins need to be recycled back to their

resident membrane to be reused. Moreover, in some cases, some SNARE proteins get mislocalized and must be retrieved back to their correct resident membrane. Therefore, SNARE protein trafficking/recycling is a very important process for cells to maintain normal cellular function (Black and Pelham, 2001).

Multiple studies have suggested that clathrin adaptor proteins are involved in the trafficking of SNAREs. Earlier studies revealed that the vesicle-associated membrane SNARE protein Vamp4 contain recognizable di-leucine motif that can bind to the AP1 adaptor complex and this di-leucine motif is required for Vamp4 localization (Peden *et al.*, 2001). But studies in different organisms have given conflicting results for another vesicle SNARE protein, Vamp7. It has been also reported that in *Dictyostelium* cells AP2 and AP3 regulate the localization of Vamp7 through di-leucine motif, a known clathrin adaptor protein binding motif. Later sequence analysis revealed that Vamp4 and Vamp7 might be the only two SNARE proteins carrying this clathrin adaptor binding motifs. On the other hand, another study revealed that the longin domain of human Vamp7, which does not contain any standard adaptor binding motif including the di-leucine motif, is actually responsible for the SNARE protein's localization and AP3 binding (Martinez-Arca *et al.*, 2003; Bennett *et al.*, 2008).

This raises the question of how SNAREs interact with clathrin associated proteins. There are two possibilities, one is that SNARE proteins interacts with clathrin adaptor proteins in a non-traditional manner, such as the binding between Vamp7 longin domain and AP3. The second possibility is that SNAREs interact directly with some non-conventional cargo binding adaptor proteins, such as AP180/CALM and epsin. Many studies have suggested the second possibility. In both *Drosophila* and *C.elegans*, clathrin assembly protein AP180 is required for recycling the SNARE synaptobrevin back to synaptic vesicles (Nonet *et al.*, 1999; Bao *et al.*, 2005). CALM, the AP180 non-

neuron homologue is also important for the endocytosis of synaptobrevin 2 (Vamp2) in mammalian cell culture (Harel *et al.*, 2008). Using crystallography, another non-conventional cargo binding protein, epsinR, was shown to bind t-SNARE protein Vti1 helical H_{abc} domain mediated through a surface-surface interaction (Miller *et al.*, 2007). Additionally, a recent study with mammalian Vamp7 revealed an interesting result that the ARF GAP Hrb, a clathrin and AP2 binding protein, is required for Vamp7 localization. In the same study, crystal structures revealed that the Hrb unstructured C-terminal tail wraps around the Vamp7 longin domain (Pryor *et al.*, 2008). However, those two crystallography studied also point out that the interactions of Vamp7/Hrb and Vti1/epsinR are thought to be unique for the specific SNARE-adaptor protein combination. This uniqueness of the interaction between clathrin components and SNARE proteins highlights the complexity and importance of the related research.

1.9 THE GOAL OF MY PH.D STUDY

As a central clathrin adaptor protein, AP2 complex binds to multiple components during clathrin mediated endocytosis including other clathrin adaptor proteins, clathrin, plasma membrane lipids and transmembrane cargos. In addition, AP2 is required for clathrin mediated endocytosis and targeting clathrin onto plasma membrane in complex systems. Moreover, in *Dictyostelium* cells, studies have shown that clathrin and clathrin adaptor proteins including AP180, epsin and Hip1r also play very important roles in other cellular processes, such as in osmoregulation, developmental cycles and cytokinesis. However whether AP2 also functions in these cellular pathways has not been assessed. Therefore the goal of the first part of my study was to understand the contribution of AP2 not only to membrane clathrin recruitment but also to other clathrin related cellular processes. This will help to further evaluate the role of AP2 as a clathrin adaptor

protein. My hypothesis was that AP2 contributes to the recruitment of plasma membrane associated clathrin and also functions in other clathrin related cellular process.

Clathrin coated vesicles have been found on different internal organelles, including the *Dictyostelium* contractile vacuole. But the composition and the function of these contractile vacuole-associated clathrin coated vesicles have not been well studied. AP180, a clathrin adaptor protein is also observed on the contractile vacuole along with clathrin and deleting AP180 caused an enlarged contractile vacuole phenotype. Additionally, AP180 is also important to the proper localization of a SNARE protein, synaptobrevin, in *C.elegans*, *Drosophila* and mammalian cells. But how AP180 controls the contractile vacuole size and whether this function is related to synaptobrevin had not been studied. Therefore the goal for the second part of my study was to investigate the function of these clathrin coated vesicles on contractile vacuoles, including the relationship of AP180 and synaptobrevin. I hypothesized that AP180 controls contractile vacuoles by trafficking a synaptobrevin *Dictyostelium* homologue at contractile vacuoles.

Chapter 2: Clathrin Adaptor Protein AP2 Complex in *Dictyostelium* Clathrin Related Processes

2.1 INTRODUCTION

Considered a central clathrin adaptor and clathrin assembly protein, the importance of AP2 has been highlighted by different experimental approaches. In HeLa cells, RNAi knockdown of various subunits of AP2 decreases the association of clathrin with the plasma membrane by 10 fold and inhibits the endocytosis of the transferrin receptor and EGF receptor but not LDL receptors (Hinrichsen *et al.*, 2003; Motley *et al.*, 2003). However, a different study using a method favoring clathrin-independent uptake showed that the internalization of EGF receptor is not AP2-dependant (Huang *et al.*, 2004). In mice, homozygous mutants in the μ2 subunit are embryonic lethal at an early stage (Mitsunari *et al.*, 2005). In *C. elegans* oocytes RNAi depletion of either the α or β2 subunit results in defects in endocytosis and the resulting mutant embryos are inviable (Grant and Hirsh, 1999). *D. melanogaster* carrying an α subunit mutation die at the pupae stage. The synapses of these mutant embryos lack vesicles and plasma membrane coated pits (Gonzalez-Gaitan and Jackle, 1997).

Interestingly, there is also evidence showing that AP2 is not always an essential clathrin adaptor protein. In *S. cerevisiae*, disruption of AP2 subunits has no effects on endocytosis (Huang *et al.*, 1999; Yeung *et al.*, 1999). In a wide variety of organisms, other clathrin adaptor proteins, such as epsin, AP180/CALM, β-arrestin, Hip1, Dab2, and ARH can all bind to both clathrin and plasma membrane (Gaidarov *et al.*, 1999; Ford *et al.*, 2001; Mishra *et al.*, 2001; Ford *et al.*, 2002; Mishra *et al.*, 2002). Some of these proteins may also have the ability to recognize their specific cargo in the absence of an intact AP2 complex. Epsin, for example, has ubiquitin-interacting motifs (UIMs) that

may help internalize the ubiquitinated EGF receptor. Knocking down AP180/CALM in HeLa cells affects the internalization of EGF receptors. Additionally, Dab2 and ARH, can recognize the NPXY motifs LDL receptor family, suggesting other clathrin adaptor proteins can recognize endocytosis cargo and recruit clathrin onto plasma membrane in AP2-independent ways (Morinaka *et al.*, 1999; Bonifacino and Traub, 2003). Taken together, AP2 may not be the only adaptor protein that recruits cargo into clathrin coated vesicles, especially in some organisms.

Previous studies done in our lab using *Dictyostelium discoideum* as a model system revealed that clathrin is involved in other important cellular pathways. Clathrin heavy chain null *Dictyostelium* are unable to perform pinocytosis. Clathrin heavy chain null cells are missing contractile vacuoles and swell in hypotonic solutions while clathrin light chain null cells have enlarged contractile vacuoles. In addition, clathrin heavy chain null cells have severe cytokinesis defects and become multinucleated when grown in suspension culture. Moreover, clathrin is required for *Dictyostelium* cell development. The *Dictyostelium* developmental cycle is initiated under conditions of starvation. During the developmental phases about 100,000 nutrients deprived vegetative *Dictyostelium* cells aggregate and differentiate, form a multicellular organism which ultimately becomes a fruiting body made up of spores on top of a stalk. Clathrin heavy chain mutant cells are not able to initiate the development cycle (O'Halloran and Anderson, 1992b; Niswonger and O'Halloran, 1997b, 1997a; Gerald *et al.*, 2001; Wang *et al.*, 2003).

Multiple clathrin accessory proteins have been previously implicated in some of the above clathrin related processes in *Dictyostelium* cells. AP180 and AP1 null cells are both osmosensitive. Furthermore, epsin null cells and Hip1r null cells make round fragile spores instead of the typical robust ovoid spores made by wild type *Dictyostelium*

cells during development (Lefkir *et al.*, 2003; Stavrou and O'Halloran, 2006; Repass *et al.*, 2007; Brady *et al.*, 2008). However, as the central clathrin adaptor protein, whether AP2 contributes to these clathrin related cellular processes remained unsolved.

I will demonstrate in this chapter that in *Dictyostelium* cells, AP2 is required for the localization of a significant amount of the clathrin (~40%) at the plasma membrane in the wild type strain Ax2. In addition, I will provide evidence that AP2 also plays roles in both cytokinesis and cell developmental cycles. How AP2 functions in cell osmoregulation will be discussed in more detail in Chapter 3.

2.2 RESULTS

2.2.1 Identification of *Dictyostelium* AP2

A BLAST search of the *Dictyostelium* genome with the human AP2α subunit gene sequence identified the closest related protein: the *Dictyostelium* homologue of AP2 α-subunit gene, *AP2A1*. The full length *Dictyostelium AP2A1* gene encoded a protein with a predicted open reading frame of 943 amino acids with a predicted molecular weight of 105 kDa. The predicted sequence shared 48% identity of amino acid sequence with the human AP2α subunit.

To determine whether *Dictyostelium* AP2 also shared functional similarity with mammalian AP2, I first examined the cellular localization of AP2 in *Dictyostelium* cells. To do this, I raised a polyclonal antibody against the α subunit for immunofluorescence staining of AP2. In mammalian cells, AP2 localizes mainly at the plasma membrane and colocalizes significantly with clathrin at the plasma membrane (Hinrichsen *et al.*, 2003; Motley *et al.*, 2003). Similarly, wild type *Dictyostelium* cells stained with anti-AP2α subunit revealed that AP2 localized to strong punctate spots at the cell membrane and weakly in the cytoplasm (Figure 2.1A top panel). Neither the membrane-associated nor the cytoplasmic puncta were seen in AP2 null cells (bottom panel). To examine whether AP2 was associated with clathrin, I then stained *Dictyostelium* cells expressing Green Fluorescent Protein (GFP) tagged clathrin light chain with the same anti-α subunit antibody. AP2 positive puncta colocalized extensively with clathrin on the cell membrane but not with clathrin puncta in the cytoplasm (Figure 2.1B). Occasionally there were clathrin puncta on the plasma membrane that were not labeled with AP2. I quantified 244 clathrin labeled puncta at the periphery of 25 cells and found that 208 (85%) clathrin puncta colocalized with AP2 while 36 (15%) peripheral clathrin puncta

were not AP2 positive. Based on sequence homology and its localization I concluded that AP2A1 was the *Dictyostelium* homologue of AP2α subunit.

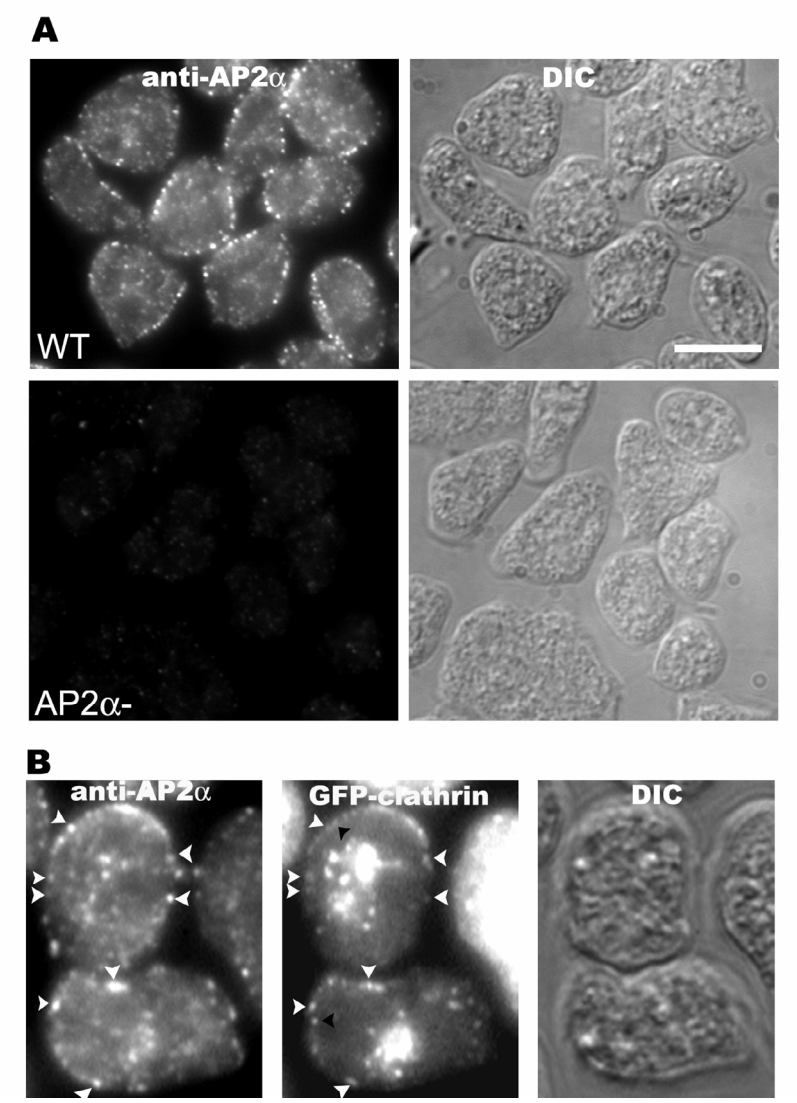


Figure 2.1 (A). Localization of *Dictyostelium* AP2 in wild type cells. Wild type cells were stained with anti-α subunit antibody (top panel). *Dictyostelium* AP2 localized to strong punctate spots at cell membrane and weakly in the cytoplasm. Neither the membrane-associated nor the cytoplasmic puncta were seen in AP2α null cells (bottom panel) (B). *Dictyostelium* AP2 colocalized with clathrin at the plasma membrane. Wild

type cells expressing GFP-CLC (clathrin light chain) stained with anti- α subunit antibody. Arrows indicate the co-localization between AP2 and clathrin at the cell peripheral. Scale bar, 10μm.

2.2.2 The AP2 complex loses its ability to associate with clathrin or the plasma membrane in the α subunit null cells.

To further explore the function of AP2, I used homologous recombination to completely delete the whole α subunit gene, *AP2A1*, one of the two large subunits of AP2 in *Dictyostelium* Ax2 wild type cells. Western blots probed with anti-α subunit antibody confirmed the absence of the α subunit in the AP2α subunit null (AP2 α-) cell line that I generated (Figure 2.2A). To establish the validity of using this AP2α- cell line as an AP2 complex mutant cell line, I first addressed the question of whether the remaining subunits of the AP2 complex retained some AP2 function in the absence of the α subunit. As a test case, I examined the fate of the μ2 subunit in AP2α null cells. I constructed a plasmid expressing the μ2 subunit tagged with GFP to visualize its localization in AP2α- cells. Similar to the localization of AP2α subunit that was shown by anti-α subunit immunostaining antibody in wild type cells, the GFP- μ2 subunit fusion protein formed discrete puncta both on plasma membrane and in the cytoplasm (Figure 2.2 B). The GFP- μ2 subunit colocalized with α subunit in wild type cells which means the GFP- μ2 subunit fusion protein was able to assemble into AP2 complexes (data not shown). When I introduced this GFP- μ2 subunit fusion protein into AP2α- cells, it remained as discrete spots in the cytoplasm but lost its ability to associate with the cell periphery, suggesting a loss of binding to the plasma membrane (Figure 2.2B). In addition, I examined the ability of the incomplete AP2 complex to associate with clathrin in AP2α- cells. In wild type cells, the GFP- μ2 subunit colocalized with clathrin. Using

clathrin light chain antibody staining, I quantified 305 clathrin puncta on the periphery of 24 wild type cells expressing GFP tagged μ2 subunit using clathrin light chain antibody staining. 50% (153 out of 304 clathrin puncta in 24 cells) of those clathrin puncta colocalized with GFP- μ2. However in AP2α- cells, the association between incomplete AP2 complex and clathrin disappeared almost completely (Figure 2.2B). In AP2α- cells only 1% (5 out of 362 clathrin puncta in 23 cells) of the cell peripheral clathrin puncta were GFP- μ2 labeled. Taken together, those results indicate that the other subunits of the AP2 complex have lost their ability to bind the plasma membranes and to associate with clathrin in the α subunit null cells.

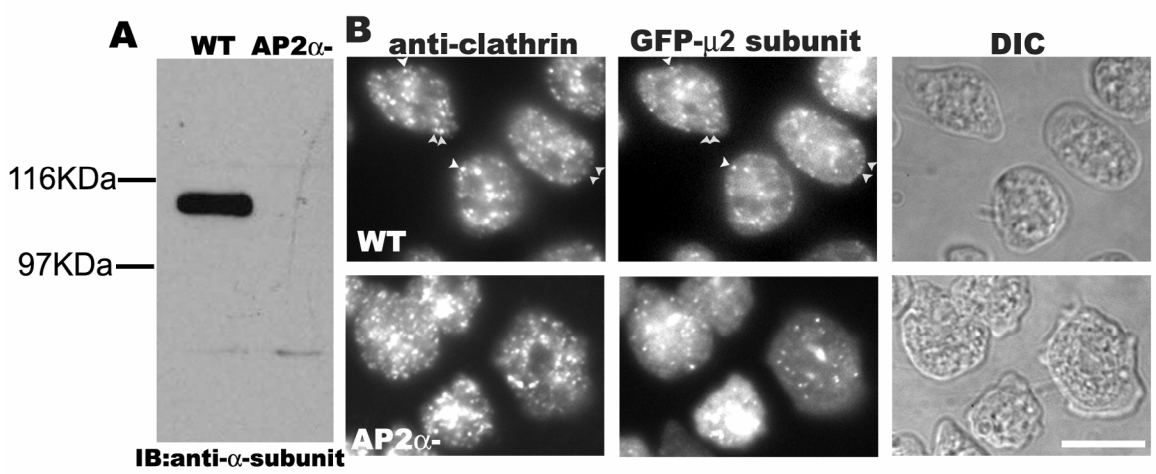


Figure 2.2 (A).Western blots probed with anti-α subunit antibody confirmed the absence of α subunit protein in the AP2α subunit null cells (AP2α-). (B). μ2 subunit in AP2α subunit null cells lost its ability to associate with either the plasma membrane or the clathrin. Both wild type cells and AP2α subunit null cells expressing GFP- μ2 subunit were stained with anti-clathrin light chain antibody (anti-clathrin). Arrows indicate the co-localization between μ2-GFP and clathrin at the plasma membrane in wild type cells. Scale bar, 10μm.

2.2.3 AP2 is responsible for recruiting ~40% of plasma membrane associated clathrin

In HeLa cells, deletion of either the α subunit or μ2 subunit abolishes the localization of almost all of the plasma membrane associated clathrin. To test whether the absence of the α subunit also alters the localization of clathrin on the plasma membrane in *Dictyostelium* cells, I labeled both wild type cells and AP2α- cells with an anti-clathrin light chain polyclonal antibody. Clathrin localized both on cell membrane and in cytoplasm in both wild type cells and in AP2α- cells (Figure 2.3A). However, compared to wild type cells, AP2α- cells had less cell periphery associated clathrin. To confirm this observation I quantified the amount of plasma membrane associated clathrin using confocal microscopy images to calculate the fluorescence intensity per plasma membrane area in 100 representative cells of both wild type cells and AP2α- cells. My data showed that in *Dictyostelium* cells there was an approximately 35%-40% (39% & 35% in two independent experiments) decrease of clathrin on plasma membrane when the α subunit was absent (Figure 2.3A). This decrease was not caused by the different clathrin expression level (Figure 2.3B). The influence of AP2 on clathrin may be cell line dependent as in a different *Dictyostelium* cell line, DH1 cells, I only observed about a 20% decrease of membrane associated clathrin in the absence of α subunit (see Chapter 3 for more detail).

The loss of ~35%-40% of clathrin on the plasma membrane raised the question of which protein(s) recruit the remaining 60% of the membrane associated clathrin in AP2α- cells. AP180/CALM and epsin are two clathrin accessory proteins that can bind clathrin and plasma membrane PI (4, 5) P2. These adaptor proteins have the potential to serve as alternative clathrin recruiters to target clathrin to the plasma membrane during endocytosis. To test that possibility, I examined the association between clathrin and

AP180 in wild type cells as well as in AP2α- cells. First I introduced GFP-tagged AP180 into AP2α- cells and stained those cells with an anti- clathrin light chain antibody. In wild type cells, AP180 colocalized with clathrin significantly (Figure 2.3C): 130 (88%) out of 147 peripheral clathrin positive puncta also labeled with GFP-AP180 in wild type cells (n=11 cells). Similarly, in AP2α- cells, AP180 was observed to colocalize normally with clathrin on the cell periphery: 181 puncta (87%) out of the 207 clathrin labeled puncta on the cell periphery colocalized with AP180 (n=15 cells). This suggests that AP180 is able to associate with clathrin on the plasma membrane in an AP2-independent way. In a separate study, epsin also has been shown to colocalize with clathrin in the absence of α subunit (Brady *et al.*, 2008).

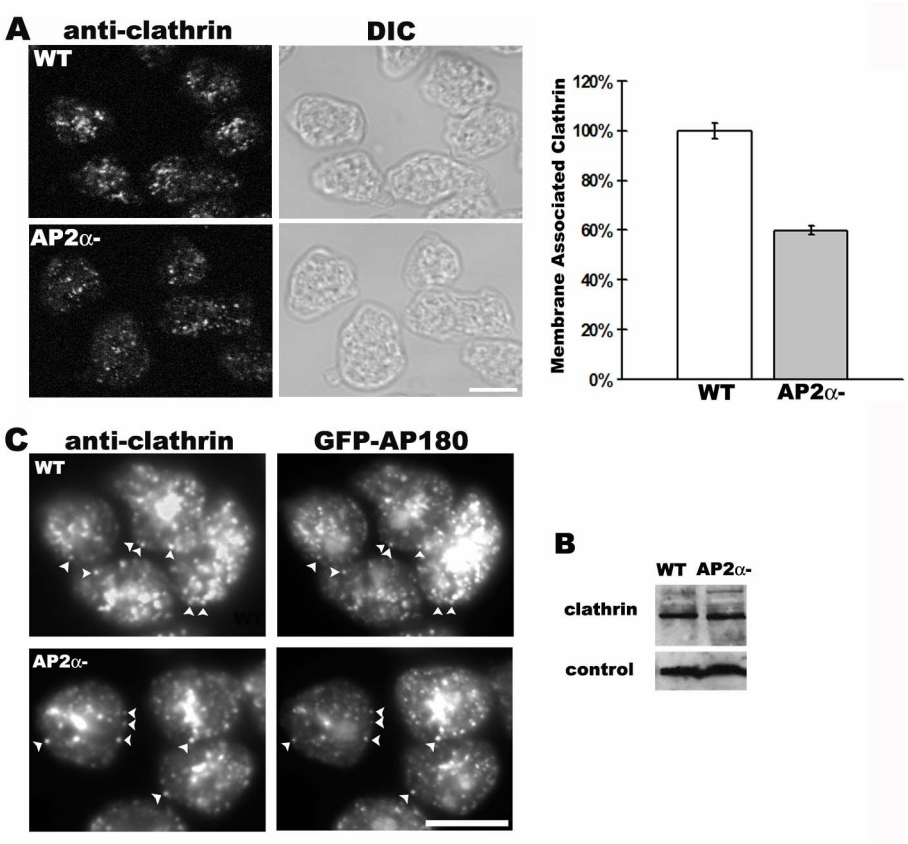


Figure 2.3 (A). AP2α null cells show 40% less membrane associated clathrin compared to wild type cells. Left: wild type Ax2 cells and α subunit null cells were stained with anti-CLC antibody. Right: top membrane associated clathrin images of both wild type cells (n=116) and AP2 α- cells (n=108) were quantified (intensity/area). Standard errors between every cell line within the same cell line were used as error bars. (B). Western blot showed that both wild type and AP2α subunit null cells have similar clathrin expression. In each lane, whole cell lysates ($1x10^6$ cells) were blotted with anti-clathrin heavy chain. Anti-Aurora antibody served as a loading control in this experiment. (C). Clathrin still colocalized with AP180 in the AP2α subunit null cells. Wild type and AP2α subunit null cells expressing GFP-AP180 were stained with anti-clathrin light chain antibody. Arrows indicate the co-localization between clathrin and AP180. Scale bar, 10μm.

2.2.4 AP2 and AP180 still co-assemble into plasma membrane puncta in the absence of clathrin

To further understand the role of AP2 during clathrin-mediated endocytosis, I examined whether clathrin is required to cluster AP2 onto the plasma membrane. In HeLa cells, localization of AP2 is not significantly different when clathrin heavy chain is knocked down by RNAi (Hinrichsen *et al.*, 2003). To observe how the absence of clathrin affects the localization of AP2 in *Dictyostelium* cells, I stained clathrin heavy chain null cells with anti α subunit antibody. In wild type cells, AP2 localized to puncta on the cell periphery and in the cytoplasm as I showed before. In clathrin heavy chain null cells, AP2 still clustered to puncta but the depletion of clathrin heavy chain abolished almost all of the cytoplasmic AP2 puncta (Figure 2.4). In wild type cells, AP2 colocalized with AP180 significantly, which indicates that AP2 co-assembled with AP180 on the plasma membrane (Figure 2.4). I quantified 240 AP2-labeled and 173

AP180-labeled cell peripheral puncta from 15 cells. 58% of the AP2 puncta colocalized with AP180 while 81% of the AP180 puncta were AP2-labeled. Interestingly, in clathrin heavy chain null cells, AP2 puncta still colocalized with AP180 on the plasma membrane. I did the same quantification in the clathrin heavy chain null cells. 76% of the 356 AP2 puncta and 89% of the 305 AP180 puncta on 21 cell peripheries colocalized with each other (Figure 2.4). The high extent of co-localization between AP2 and AP180 indicates that the association between AP2 and AP180 at the plasma membrane is clathrin-independent.

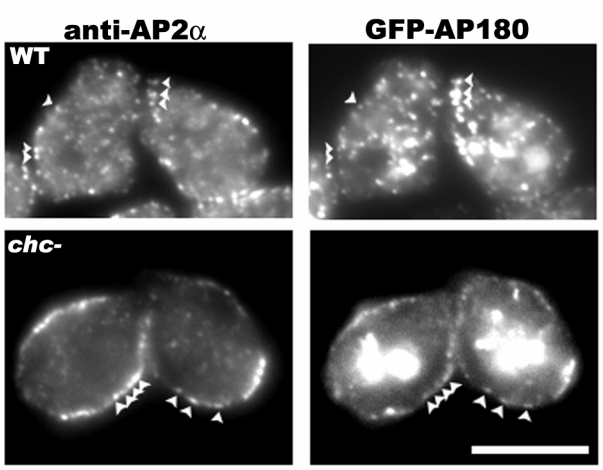


Figure 2.4 Co-localization of AP2 and AP180 in the absence of clathrin. Wild type and clathrin heavy chain null cells (*chc*- cells) expressing GFP-AP180 were stained with an anti α-adaptin antibody. AP2 and AP180 colocalized significantly in wild type cells as well as in the *chc*- cells (arrows). Scale bar, 10μm.

2.2.5 AP2α subunit null mutants have mild phenotypes in clathrin related pathways

Clathrin is implicated in *Dictyostelium* cell pinocytosis, cytokinesis in suspension, osmoregulation and the developmental cycle (O'Halloran and Anderson, 1992b;

Niswonger and O'Halloran, 1997b, 1997a). To evaluate whether AP2 also plays important roles in these clathrin related processes, I assessed each process in AP2α subunit null cells.

To test whether the AP2 complex functions in the fluid-phase endocytosis, I examined the internalization of fluid phase markers in both wild type cells and AP2α null cells. Unlike clathrin heavy chain mutant cells, AP2α- cells internalized fluid as efficiently as wild type cells (Figure2.5A).

Dictyostelium discoideum cells use two distinct cytokinesis mechanisms. When grown in suspension, *Dictyostelium* cells go through a myosin II dependent cell division; when grown on substrates they use an adhesion-dependent but myosin II-independent mechanism (Nagasaki *et al.*, 2001). Clathrin heavy chain mutant also can not complete cytokinesis when grown in suspension (Niswonger and O'Halloran, 1997b; Gerald *et al.*, 2001). When placed on a substrate, only 1% of wild type cells had more than one nucleus. But surprisingly, in AP2α- cells about 35% of the cells were multinucleate (Figure 2.5B). However, AP2α null cells did not have an apparent cytokinesis defect when placed in suspension culture. This indicates that AP2α- cells also have a myosin II-independent cytokinesis defect.

I also examined the developmental cycle of AP2α- cell lines. AP2α subunit null cells had a full development cycle and formed fruiting bodies like wild type cells but were slightly smaller (Figure 2.5C). Strikingly, during chemotaxis AP2α- cells formed multiple pseudopods while wild type *Dictyostelium* cells had only two protrusions with one leading edge and one tail. The mutant cells did form aggregation centers, but most of the aggregation centers were much smaller compared to wild type cells suggesting a possible role of AP2 in *Dictyostelium* aggregation (Figure 2.5D).

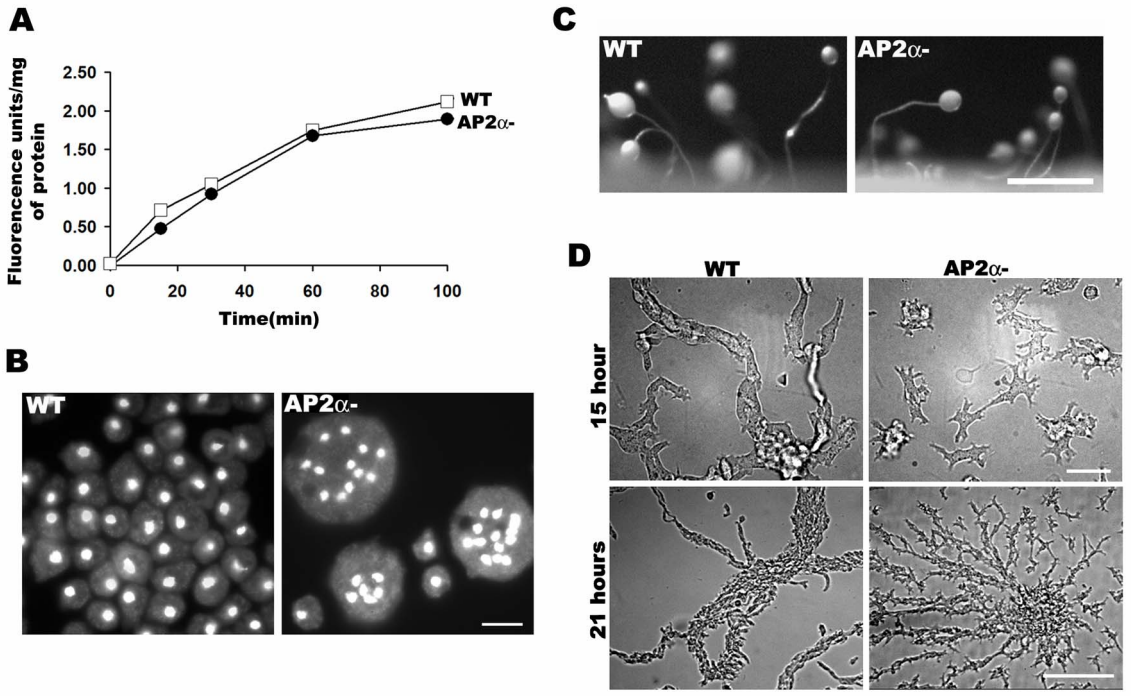


Figure 2.5 Characterizing AP2α subunit null cells in clathrin-related cellular pathways. (A). Fluid-phase endocytosis in wild type and AP2α null cells. Both wild type and AP2α null cells were incubated with 2mg/ml FITC-Dextran and the uptake of the FITC-Dextran were measured by a fluorometer. AP2α null cells (black circle) internalized the FITC-Dextran as efficiently as the wild type cells (white square). (B). AP2α null cells had multinucleated cells when grown on plate. Wild type and AP2α null cells were stained with DAPI. 35% of the AP2α null cells had more than one nucleus and only 1% of the wild type cells have more than one nucleus (n=100 cells for each cell line). Scale bar, 10μm. (C). AP2α null cells formed fruiting bodies during development. Both wild type and AP2α null cells were plated on a starvation plates and incubated at 18^0C for ~48 hrs. Both wild type cells and AP2α null cells differentiated into fruiting bodies, but the fruiting bodies made of AP2α null cells were slightly smaller. Scale bar, 0.5mm. (D). AP2α null cells formed multiple pseudopods during aggregation. Both wild type and AP2α null cells were incubated in glass chambers in PDF buffer at 18^0C for 15hrs-21hrs. At 15hrs, AP2α null cells formed multiple pseudopods while wild type cells only had one leading edge and one tail. Scale bar, 20μm. At 21hrs, wild type cells formed large

aggregation centers with long, smooth and thick cell streams, but the AP2α null cells formed smaller aggregation centers with shorter and thinner streams. Scale bar, 100μm.

2.3 DISCUSSION

In this chapter, I identified, cloned and localized the AP2α subunit from *Dictyostelium discoideum.* I found that AP2 in the *Dictyostelium* Ax2 strain is responsible for recruiting about 40% of the plasma membrane-associated clathrin. Lastly, I showed that AP2 is involved in some, but not all, clathrin-related cellular processes.

2.3.1 AP2 is important but not required for targeting clathrin onto the plasma membrane during clathrin mediated endocytosis

In *Dictyostelium* Ax2 wild type cells, AP2 is responsible for targeting around 40% of the plasma membrane associated clathrin. Moreover, other clathrin related proteins, AP180 as well as epsin, can still associate with clathrin on the plasma membrane in α-adaptin null cells (Brady *et al.*, 2008). In addition, in *Dictyostelium*, clathrin colocalized with AP2 extensively on the plasma membrane but there remained some clathrin puncta without AP2. All of these results indicate that there are alternative clathrin adaptors other than AP2, such as AP180 and epsin, that recruit clathrin onto the plasma membrane. These alternative adaptors might be responsible for recruiting the rest (60%) of the membrane clathrin in an AP2-independent way and different adaptors may be responsible for internalizing their own specific cargo. However, the influence of AP2 on clathrin recruitment could be cell line-dependent as in DH1 cells I only found a ~20% decrease of plasma membrane-associated clathrin signal in AP2α subunit null cells.

Secondly, I showed that the AP2 complex was recruited onto the plasma membrane even in the absence of clathrin. This suggests that the AP2 complex could be

targeted to the plasma membrane before clathrin. This binding between AP2 and plasma membrane could be driven by the binding force between AP2 and sorting signals of transmembrane cargo and/or binding between AP2 and plasma membrane phosphoinositide. Without clathrin, AP2 can target to the plasma membrane and can also assemble with AP180 within puncta at the plasma membrane. One possible explanation is that AP2 and other clathrin accessory protein form "pre-pits" before clathrin is recruited to the plasma membrane. However, the internalization of those "pre-pits" can not be accomplished without clathrin. Using electron microscopy (EM), this kind of clathrin deprived "pre-pits" or "sub-domains" were also observed by Hinrichsen in mammalian cells (Hinrichsen *et al.*, 2006).

After clathrin coated vesicles pinch off from the plasma membrane, AP2 dissociates from the vesicles. When I stained *Dictyostelium* wild type cells with an anti α-adaptin antibody, I observed that AP2 fluorescent signals were more intense on the plasma membrane than in the cytoplasm. This suggests that AP2 leaves the clathrin coated vesicles once the vesicles get internalized and the process of uncoating AP2 from the clathrin coated vesicles occurrs in a relatively slow and gradual way.

2.3.2 AP2 is essential for clathrin function only in multi-cellular systems but not in simple organisms

For many years, AP2 has been considered as the essential adaptor for clathrin during endocytosis. In multicellular organisms, such as *C.elegans*, *D.melanogaster* and mice, disruption of the AP2 complex results in embryonic lethality and inhibition of clathrin mediated endocytosis (Gonzalez-Gaitan and Jackle, 1997; Grant and Hirsh, 1999; Mitsunari *et al.*, 2005). In HeLa cell culture, depletion of AP2 abolished almost all of the plasma membrane associated clathrin (Hinrichsen *et al.*, 2003; Motley *et al.*, 2003).

In contrast, depletion of AP2 subunits in *S. cerevisiae*, a unicelluar system, has no effect on cell viability or endocytosis (Huang *et al.*, 1999; Yeung *et al.*, 1999). Deletion of full length AP2α subunit gene using homologous recombination in *Dictyostelium* Ax2 cells resulted in loss of only about 40% of plasma membrane associated clathrin. This *Dictyostelium* AP2 mutant cell line also showed mild defects in cytokinesis and development. AP2α subunit null phenotypes in *Dictyostelium* cells were less severe than in multi-cellular systems but more severe than in *S. cerevisiae*. Therefore in simple organisms AP2 is functionally redundant with other clathrin accessory proteins. However, in multi-cellular organism, AP2 has evolved to be the clathrin adaptor protein specialized and important for recognizing cargo and recruiting clathrin onto plasma membrane.

2.3.3 α subunit is required for the AP2 complex to bind plasma membrane

AP2 binds to plasma membrane PI (4,5) P2 through two sites on the α and μ2 subunits. I want to know if either of the two sites are independently sufficient for AP2 binding the plasma membrane. Results from tissue culture cells using an *in vitro* membrane binding assay suggest that the PIP2 binding site on the α-subunit is essential in the initial recruitment of AP2 onto the plasma membrane. The PIP2 binding site on the μ2 subunit further strengthens the association between AP2 and the plasma membrane (Honing *et al.*, 2005). In *Dictyostelium* cells, the μ2 subunit completely lost its membrane localization in the absence of α subunit which further supports that the membrane binding site on α-subunit is also crucial for targeting AP2 onto the plasma membrane *in vivo*.

2.3.4 AP2 is also involved in *Dictyostelium* cell chemotaxis

Upon starvation, around 100,000 *Dictyostelium* cells use pulse of cAMP to signal and move into an aggregate, ultimately generating a multicellular structure. During aggregation, *Dictyostelium* cells polarize by projecting a single pseudopod through actin filaments polymerization in the direction of the cAMP source (Kessin, 2001).

PI (4,5) P2 is a regulator of actin polymerization through the action of different regulatory proteins. It activates the WASP family proteins which leads to Arp2/3 complex induced actin filament polymerization (Rohatgi *et al.*, 1999). Aside from this function, PI (4,5) P2 also binds actin filament capping proteins such as CapZ and gelsolin and dissociates them from actin filaments. This dissociation can stimulate actin filament polymerization (reviewed by (Cooper and Schafer, 2000)).

AP2α subunit null *Dictyostelium* cells extended multiple pseudopods during aggregation stage. Clathrin heavy chain null cells have an increase in roundness and a reduction in polarity during chemotaxis (O'Halloran and Anderson, 1992b). Why do AP2α subunit null cells have a totally different phenotype than the clathrin mutant cells during cell aggregation? One explanation is that AP2 has two binding sites for plasma membrane PI (4,5)P2 while clathrin does not have any known plasma membrane binding sites. In AP2α subunit null cells, more free PI (4,5) P2 may be exposed on the plasma membrane. This may cause an increased amount of PI (4,5) P2 molecules available to stimulate the actin filament organization and result in the formation of multiple pseudopods in the AP2α null cells.

Alternatively, PtdIns (3,4,5) P3 is a phosphoinositide produced from PI (4,5) P2 by PI3 kinases and degraded by PtdIns (3,4,5) P3 phosphatase (PTEN). PI (3,4,5) P3 is important for recruiting many PH domain-containing proteins including those proteins essential for pseudopod formation during chemotaxis. Restricting the formation of

PtdIns (3,4,5) P3 is one important way for cells to control lateral pseudopods extension. *Dictyostelium* cells with depletion of PtdIns (3,4,5)P3 phosphatase (PTEN) form many pseudopods and move indirectly towards the chemoattractant (Funamoto *et al.*, 2002; Iijima and Devreotes, 2002). In AP2α subunit null *Dictyostelium* cells, more free PI (4,5) P2 may be exposed on the plasma membrane providing PI3 kinase with more substrate molecules to produce PI (3,4,5) P3. This may cause an abnormally high level of PI (3,4,5) P3 on the plasma membrane of AP2α subunit null cells and, under these conditions, PI (3,4,5) P3 maybe able to recruit more PH-domain proteins which results in an increased number of pseudopods. Either of these scenarios suggest that one novel function for AP2, suggested by my studies, is to regulate the amount of free PI (4,5) P2 that is exposed on the plasma membrane.

These possibilities are not the only explanation. Many other proteins contribute to *Dictyostelium* chemotaxis, including Protein Kinase B, PI3 kinase, p21-activated kinase (PAK) (reviewed by (Kimmel and Parent, 2003)). If AP2α subunit is involved in regulating any of these proteins, then the absence of the α subunit may cause the multiple pseudopod phenotype during aggregation. Therefore how AP2 regulates *Dictyostelium* pesudopods number needs to be further studied.

Chapter 3 Study of Clathrin Coated Vesicles on *Dictyostelium* Contractile Vacuole Complex

3.1 INTRODUCTION

Eukaryotic cells internalize both receptors and nutrients from the plasma membrane through clathrin coated vesicles. During endocytosis, receptors concentrate within clathrin coated vesicles via interactions with different clathrin adaptors. In addition to binding specific receptors, clathrin adaptors, or clathrin accessory proteins, also promote clathrin assembly on membranes. After the clathrin coat is assembled, the coated vesicle buds and pinches off from the plasma membrane. The internalized cargo is subsequently transported to endosomes or recycling compartments (reviewed by (Kirchhausen, 2000; Brodsky et al., 2001; Mousavi et al., 2004; Royle, 2006)).

The physiological contributions of clathrin adaptors and accessory proteins are diverse and some are well documented. AP2 (a tetrameric AP family member), epsin and AP180/CALM assemble clathrin triskelia into lattices of hexagons and pentagons on the plasma membrane (Ahle and Ungewickell, 1986; Keen, 1987; Prasad and Lippoldt, 1988; Lindner and Ungewickell, 1992; Ye *et al.*, 1995; Ye and Lafer, 1995; Hirst and Robinson, 1998; Hao *et al.*, 1999; Ford *et al.*, 2002; Owen, 2004; Edeling *et al.*, 2006; Rodemer and Haucke, 2008). In addition to their clathrin assembly ability, AP2, AP180/CALM and epsin also have additional distinct roles. AP2 recognizes discrete sorting signals formed from peptide motifs on transmembrane cargo (Ohno *et al.*, 1995; Owen and Evans, 1998; Owen *et al.*, 2001; Boll *et al.*, 2002; Kelly *et al.*, 2008). AP180/CALM is implicated in the efficient assembly of clathrin cages of uniform size (Ahle and Ungewickell, 1986; Prasad and Lippoldt, 1988; Ye and Lafer, 1995; Zhang *et al.*, 1998; Nonet *et al.*, 1999; Ford *et al.*, 2001; Ford *et al.*, 2002). Epsin can induce

plasma membrane curvature during vesicle invagination *in vitro* (Itoh *et al.*, 2001; Ford *et al.*, 2002). Sla2/Hip1 family members also play an important role during clathrin mediated endocytosis and serve as linkers between clathrin and F-actin (McCann and Craig, 1997; Wesp *et al.*, 1997; Engqvist-Goldstein *et al.*, 1999; Yang *et al.*, 1999; Engqvist-Goldstein *et al.*, 2001; Itoh *et al.*, 2001; Mishra *et al.*, 2001; De Camilli *et al.*, 2002; Henry *et al.*, 2002; Legendre-Guillemin *et al.*, 2004; Brett *et al.*, 2006).

In addition to the plasma membrane, clathrin coated vesicles are observed on internal organelles, including the trans-Golgi network (TGN) and endosomes (Friend and Farquhar, 1967; Stoorvogel et al., 1996). At the TGN, clathrin coated vesicles transport lysosomal hydrolases to endosomes (Friend and Farquhar, 1967; von Figura and Weber, 1978; Gonzalez-Noriega et al., 1980). The endosome associated clathrin coated vesicles are involved in sorting Shiga toxin from late endosomes to the TGN (Lauvrak *et al.*, 2004). In addition to transporting cargo to the TGN, endosome-associated clathrin coated pits have been shown to function in the recycling of transferrin receptors back to the plasma membrane (van Dam and Stoorvogel, 2002). As with coated pits on the plasma membrane, clathrin associated proteins also found on coated vesicles that originate from internal organelles. Monomeric GGAs and the tetrameric AP family member AP1 serve as adaptor proteins and bind sorting signals on TGN membrane receptors (Austin *et al.*, 2002; Crottet *et al.*, 2002; Mishra *et al.*, 2002; Shiba *et al.*, 2002). AP1 also localizes onto endosomes when retrograde transport between Golgi apparatus and endosomes is blocked (Mallard *et al.*, 1998). With its ability to promote clathrin assembly, AP1 probably also serves as a clathrin assembly protein on TGN and endosomes (Keen, 1987). The AP family member AP3 may play a role on the endosomes; but whether AP3 is related to clathrin function is still under debate (reviewed by (Robinson and Bonifacino, 2001) and (Nakatsu and Ohno, 2003)). Recent studies in tobacco cells,

revealed that clathrin localized to another internal cell organelle, the phragmoplast during the late stage of cell division(late anaphase and telophase) (Tahara *et al.*, 2007). The localization of clathrin on the phragmoplast suggests a possible role for clathrin in the organization of the phragmoplast in cytokinesis plant cells. To date, how clathrin accessory proteins contribute to coated vesicle function on internal organelles is much less understood than with plasma membrane coated pits.

Contractile vacuoles are internal organelles found in protists that are important for osmoregulation (reviewed by (Allen and Naitoh, 2002)). In *Dictyostelium* cells, the contractile vacuole is formed from a dynamic labyrinth of membranous tubules and bladders (cisternae) that interconnect in a complex network. In hypo-osmotic conditions, contractile vacuoles collect excess water through tubules which rounds up to bladders that subsequently fuses with plasma membrane and contracts to expel the water into the extracellular space (Gerisch *et al.*, 2002).

Clathrin puncta have been found on *Dictyostelium* contractile vacuoles (Heuser, 2006; Stavrou and O'Halloran, 2006). Clathrin also contributes to *Dictyostelium* contractile vacuole function as clathrin light chain mutant null cells display abnormally large and dysfunctional contractile vacuoles while clathrin heavy chain mutants contain a dispersed contractile vacuole system (O'Halloran and Anderson, 1992b; Wang *et al.*, 2003). Clathrin assembly proteins, AP180 and AP1, are also linked to contractile vacuole function. AP180 labels *Dictyostelium* contractile vacuoles and AP180 null cells display abnormally large contractile vacuoles (Stavrou and O'Halloran, 2006). AP1 was not found on the contractile vacuole but AP1 μ1 subunit null mutants are osmosensitive (Lefkir *et al.*, 2003). At present it is not clear how these clathrin accessory proteins contribute to contractile vacuole function, or how AP180 and clathrin limit the size of contractile vacuoles.

In this study, I found that clathrin coated vesicles on contractile vacuole bladders contain adaptor proteins AP180, AP2 and epsin but not Hip1r. I also identified an interaction between AP180 and a contractile vacuole SNARE, Vamp7B. These results suggest a mechanism how AP180 and coated vesicles contribute to size regulation of contractile vacuoles by regulating the internal distribution of fusion-competent SNARE proteins.

3.2 REUSLTS

3.2.1 Clathrin coated vesicles on the contractile vacuoles contain AP2, AP180, epsin but not Hip1r

To explore the diversity of clathrin adaptors on the contractile vacuole I developed reagents that allowed us to determine the distribution of clathrin, AP2, AP180, epsin and Hip1r in *Dictyostelium* (Materials and Methods) and (Stavrou and O'Halloran, 2006; Repass *et al.*, 2007; Brady *et al.*, 2008). To compare the distribution of these clathrin adaptors on the contractile vacuole versus the plasma membrane I performed double and triple staining immunofluorescence experiments on wild type *Dictyostelium* cells. Conceivably clathrin could assemble on membranes by using either assembly protein AP180 or AP2. To determine whether individual clathrin punctae contain one or both of these assembly proteins, I did a triple staining experiment. Cells co-expressing Red Fluorescent Protein-tagged clathrin light chain (RFP-CLC) and Green Fluorescent Protein-tagged AP180 (GFP-AP180) were immunostained with our anti-α subunit polyclonal antibody and secondary antibody conjugated with Pacific Blue. We found that 78% (n=221 clathrin punctae on 27 cells) of plasma membrane associated clathrin punctae contained AP2, and 69% of these clathrin punctae contained both AP2 and

AP180. This extensive co-localization indicated that the majority of plasma membrane clathrin coated vesicles have both AP2 and AP180 (Figure 3.1A).

I also examined the composition of assembly protein in clathrin coated vesicles on *Dictyostelium* contractile vacuoles. To enhance contractile vacuole activity, I immersed cells immersed in water, and then fixed them for immunofluorescence microscopy. Cells expressing RFP (red fluorescent protein)-tagged clathrin light chain and GFP-tagged AP180 were fixed and stained with anti-α subunit antibody followed by a secondary antibody conjugated with Pacific Blue. I found that similar to plasma membrane, 85% of clathrin punctae (n=67 clathrin punctae on 13 contractile vacuoles) on contractile vacuoles were labeled with AP2, and 82% of these clathrin punctae contained both AP2 and AP180 (Figure 3.1B).

The presence of AP2 and AP180 on clathrin coated vesicles raised the question of whether clathrin coated vesicles on the contractile vacuoles contained all the clathrin accessory proteins normally found at the plasma membrane. I therefore investigated whether epsin or Hip1r are also on contractile vacuoles. I co-expressed RFP-tagged epsin with GFP-AP180 in wild type cells and stained these cells with an antibody against the contractile vacuole marker Rh50 (Benghezal *et al.*, 2001). I found that epsin is also found on the contractile vacuole and colocalized with AP180 (Figure 3.1C). To determine whether Hip1r localizes on contractile vacuoles, I stained wild type cells expressing GFP-AP180 with an anti-Hip1r polyclonal antibody. In contrast with epsin, Hip1r was absent from contractile vacuoles despite the presence of AP180 (Figure 3.1D).

My data suggested that the contractile vacuole is an unusual organelle in that it contains three of the clathrin adaptors normally associated with the plasma membrane: AP2, AP180 and epsin. In contrast, Hip1r was only on the plasma membrane but not on contractile vacuoles. This result suggested that clathrin coated vesicles on the

contractile vacuoles have a similar, but not identical, composition to coated vesicles on the plasma membrane.

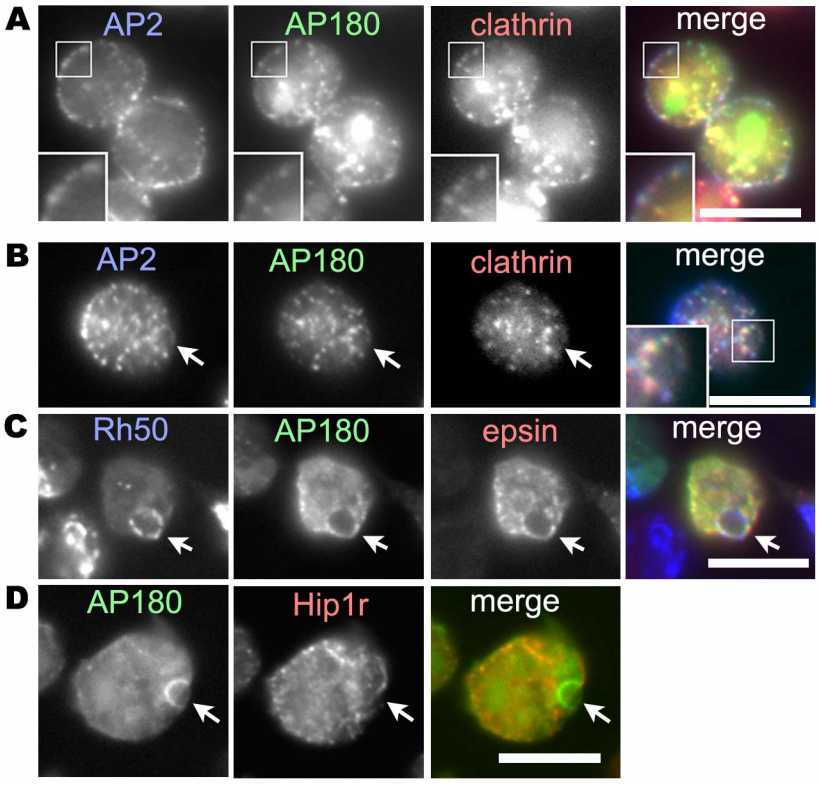


Figure 3.1. (A,B) The majority of clathrin punctae on the plasma membrane and the contractile vacuole contain both AP2 and AP180. Wild type cells expressing GFP-AP180 (green) and RFP-clathrin light chain (red) were immunostained for AP2α subunit (blue). (C) Epsin localizes on GFP-AP180-labeled contractile vacuoles. Wild type cells expressing GFP-AP180 (green) and epsin-RFP (red) were immunostained with a contractile vacuole marker, Rh50 (blue). (D) Hip1r does not localize on AP180-labeled contractile vacuoles. Wild type cells expressing GFP-AP180 (green) were immunostained for Hip1r (red). Contractile vacuoles in (B, C, D) are indicated by arrows. Scale bar, 10 μm.

3.2.2 Generation of AP2α subunit null, AP180 null, epsin null, AP2α/AP180 double null, AP2α/epsin double null cell line in *Dictyostelium* DH1 cell line background

In Chapter 2, I described how I generated an AP2α subunit null cell line in *Dictyostelium* Ax2 wild type cell background. To further study the role of AP2, AP180 and epsin in *Dictyostelium* contractile vacuole, I decided to generate AP2α/AP180 double mutant and AP2/epsin double mutant cell lines in addition to single mutant cell lines. Because of the limited selective markers in the Ax2 background, I switched to a different *Dictyostelium* DH1 wild type cell line with more selective markers. I first generated an AP2α subunit null cell line in DH1 cells through homologue recombination utilizing the same construct I used to generate the AP2α subunit null cells in Ax2 cells. AP2α subunit/AP180 double mutant cell line (AP2α/AP180 DKO) and AP2α/epsin double mutant cell line (AP2α/epsin DKO) were generated by further deleting AP180 or epsin in the α subunit null cell line using the same method as generating the single mutant cell lines in previous studies (Stavrou and O'Halloran, 2006; Brady *et al.*, 2008). The AP180 single null, epsin single null cell lines in DH1 cells were generated by Irene Stavrou and Rebecca Brady respectively. The absence of the products of the AP2α subunit, AP180 or epsin genes was confirmed using western blot analysis (Figure 3.2 A and B).

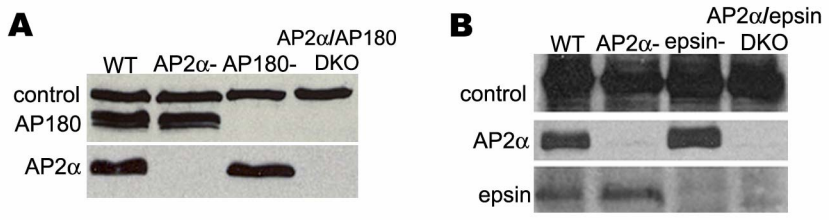


Figure 3.2 Generation of mutant cell lines. (A) An immunoblot of whole cell lysates of the wild type (WT) and AP2α subunit null (AP2α-), AP180 null (AP180-), AP2α subunit

and AP180 double null (AP2α/AP180 DKO) stained with anti-AP2α subunit and anti-AP180 antibodies. A non-specific protein band recognized by anti-AP180 antibody was used as a loading control. (B) An immunoblot of whole cell lysates of the wild type (WT) and AP2α subunit null (AP2α-), epsin null (epsin-), AP2α subunit and epsin double null (AP2α/epsin DKO) stained with anti-AP2α subunit and anti-epsin antibodies. Anti-myosin-heavy-chain (MHC) antibody was used as a loading control.

3.2.3 Depletion of AP2 and AP180 cause synergistic defects in osmoregulation

To investigate whether AP2, AP180 and epsin contribute to contractile vacuole function, we generated single mutants in AP2 (α-subunit), AP180, and epsin. I also generated AP2α/AP180 double mutant and AP2α/epsin double mutant cell lines. Inspection of these cell lines in a hypotonic environment revealed that they all contained prominent contractile vacuoles. This is in contrast to clathrin heavy chain null cells or AP1 (μ1 subunit) null cells that lack large contractile vacuoles (Lefkir *et al.*, 2003) . Thus, while clathrin and AP1 play a role in the biogenesis of the contractile vacuole, clathrin and the other adaptors could contribute a separate function on contractile vacuoles after they have formed.

It has been showed previously that loss of clathrin light chain or AP180 leads to enlarged contractile vacuoles (Wang *et al.*, 2003; Stavrou and O'Halloran, 2006). To investigate whether loss of AP2 and epsin also influences contractile vacuole size, I measured contractile vacuoles in various mutant cell lines in two different osmotic environments (Figure 3.3A). In isotonic nutrient medium, the maximum average diameter of contractile vacuoles in wild type cells was 3.39±0.10 μm (n=32 contractile vacuoles). Epsin null cells displayed contractile vacuoles of similar size (3.41±0.15μm, n=20 contractile vacuoles). In contrast, AP2α subunit null, AP180 null and the

AP2α/AP180 DKO cells had enlarged contractile vacuoles (AP2α-,3.59 ±0.14 μm, n=21 contractile vacuoles; AP180-, 4.05 ±0.20 μm, n=28 contractile vacuoles, AP2α/AP180 DKO, 4.88±0.22 μm, n=24 contractile vacuoles). Among all the mutant cells, AP2α/AP180 DKO cells displayed the largest contractile vacuoles whereas AP2α/epsin DKO cells had contractile vacuoles similar in size to those in AP2α single null cells (3.70±0.14μm,n=21 contractile vacuoles) (Figure 3.3B).

In water, cells displayed similar defects in their contractile vacuoles. Contractile vacuoles in wild type cell and epsin null cells reached a similar maximum size (wild type, 3.48 ±0.09 μm, n=52 contractile vacuoles; epsin-, 3.36±0.07 μm, n=57 contractile vacuoles). However, AP2 and AP180 mutants displayed larger contractile vacuoles (AP2α-,3.99 ±0.13 μm, n=40 contractile vacuoles; AP180-, 4.24 ±0.09 μm, n=61 contractile vacuoles; AP2α/AP180 DKO, 5.20 ±0.11 μm, n=56 contractile vacuoles). While the AP2α/AP180 DKO cells in hypo-osmotic environment exhibited the largest contractile vacuoles AP2α/epsin DKO displayed contractile vacuole similar in size to AP2A1α single null cells with a size of 3.91±0.12μm (n=45 contractile vacuoles) (Figure 3.3B).

Thus, among all clathrin adaptors, only the loss of AP2 or AP180 contributed to contractile vacuole size. Loss of epsin did not affect contractile vacuole size in single or double mutants. Since the AP2α/AP180 double null mutant exhibited an enhanced contractile vacuole size phenotype relative to the single mutants, I postulated that AP180 and AP2 contributed individual functions in controlling contractile vacuole size. Therefore, we explored in more detail the role of AP2 and AP180 on contractile vacuole function.

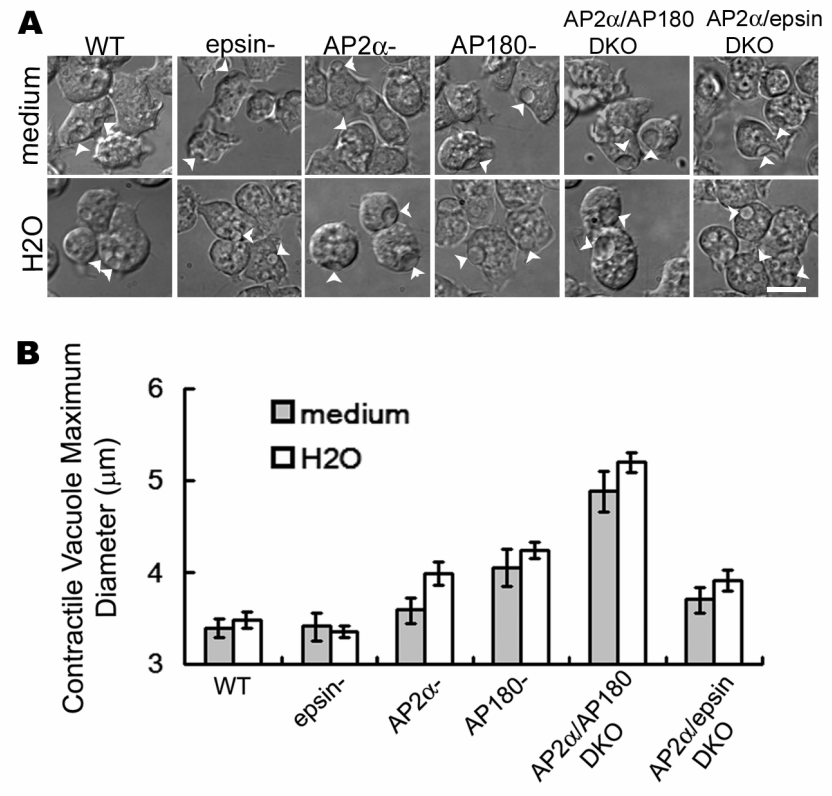


Figure 3.3 AP2 and AP180 mutant cell lines have enlarged contractile vacuoles. (A) DIC images of wild type (WT), epsin null (epsin-), AP2α subunit null (AP2α-), AP180 null (AP180-), AP2α subunit and AP180 double null (AP2α/AP180 DKO), AP2α subunit and epsin double null (AP2α/epsin DKO) cell lines in both isotonic medium (top panel) and in water (bottom panel). Arrowheads indicate contractile vacuoles. Scale bar, 10 μm. (B) Quantification of contractile vacuole diameters in both isotonic (medium) and hypotonic condition (H2O). Error bar, standard error; n=20-61 contractile vacuoles for each condition.

3.2.4 Both AP2 and AP180 recruit clathrin onto contractile vacuoles

To explore the contribution of AP2 and AP180 to clathrin recruitment onto the contractile vacuoles, Irene Stavrou, a former lab member imaged living cells in water that

expressed GFP-tagged clathrin light chain (CLC). In wild type cells, AP2α null and AP180 null cells, she frequently observed punctae of clathrin that outlined the circumference of the bladder and remained until the bladder discharged. However, clathrin punctae were rarely found on the contractile vacuoles in AP2α/AP180 DKO cells. I then quantified how many contractile vacuole bladders were labeled with clathrin. I scored a contractile vacuole as clathrin-positive if at least one clathrin punctae associated with the bladder for at least 9 seconds during its lifetime, a method has been used by Stavrou in 2006 (Stavrou and O'Halloran, 2006). In wild type cells, 56% contractile vacuoles were clathrin positive. In the AP2α null and AP180 null, I observed a decrease in clathrin-labeled contractile vacuoles (30% contractile vacuole in AP2α cells and 35% in AP180 null were labeled by clathrin, n=20 cells) (Figure 3.4A). In AP2α/AP180 DKO cells, clathrin was associated with only 15% (n=20 cells) of their contractile vacuoles (Figure 3.4A). These differences in the association of clathrin with contractile vacuoles were not caused by different expression level of clathrin construct (Figure 3.4B). Therefore, the enhanced phenotype in clathrin recruitment in the double mutant further suggests that both AP2 and AP180 contribute to the recruitment of clathrin to the contractile vacuole.

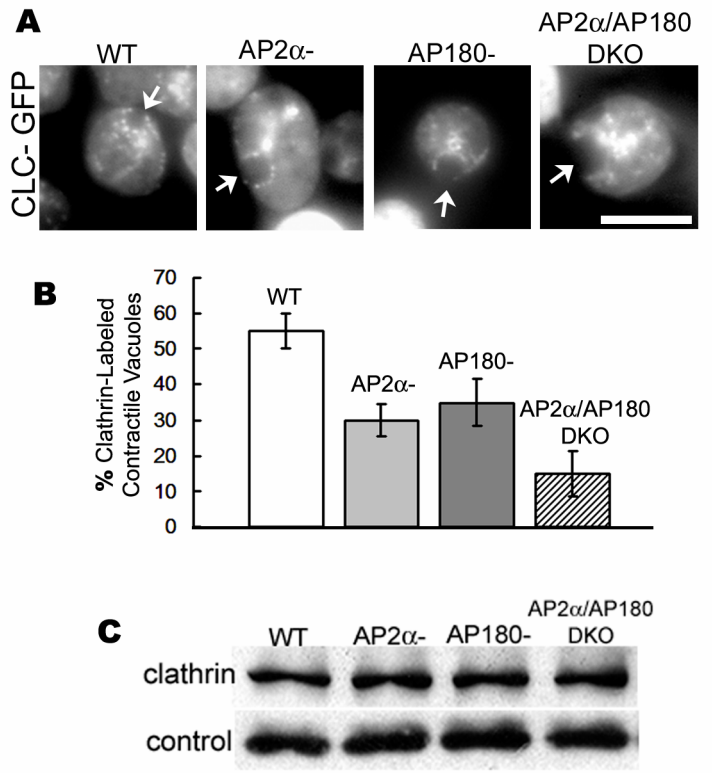


Figure 3.4 The association of clathrin at the contractile vacuole is reduced in the absence of AP2α subunit and/or AP180. (A) Living wild type, AP2α-, AP180- and AP2α/AP180 DKO cells expressing GFP-clathrin light chain in hypotonic condition (water) were imaged using fluorescence microscopy. GFP-CLC punctae decorated some number of contractile vacuoles in all four cell lines (arrows). Scale bar, 10μm. (B) Quantification of clathrin labeled contractile vacuoles in wild type, AP2α-, AP180- and AP2α/AP180 DKO cells. Error bar, standard error, n=20 cells for each cell line. (C) Western blots show equivalent expression levels of clathrin in all four cell lines. In each lane, whole cell lysates ($1x10^6$ cells) were blotted with anti-clathrin heavy chain. Anti-Aurora antibody served as a loading control in this experiment.

3.2.5 Loss of AP180 but not AP2 causes an increase in fusion among contractile vacuoles

To determine how the loss of AP2 and AP180 influenced the formation of enlarged contractile vacuoles, we monitored the dynamic behavior of contractile vacuoles in different cell lines by Differential Interference Contrast (DIC) microscopy. In wild type cells, contractile vacuoles became round as they filled, reached a maximum size and moved to the membrane to contract and discharge their contents to the extra-cellular space. Occasionally one contractile vacuole would fuse with another and form a single larger contractile vacuole. In mutant cells, contractile vacuoles exhibited similar phases: expansion, contact with the plasma membrane, and discharge. Strikingly, I also observed that contractile vacuoles fused more frequently with each other in the AP180 single null cells and AP2α/AP180 double null cells but not in AP2α subunit null cells (Figure 3.5A and data not shown). I quantified the fusion frequency by recording how many times two contractile vacuoles fused in each cycle of contractile vacuole expansion and discharge. In both wild type cells and AP2α subunit single null cells, the fusion frequency was 0.10 event per vacuole life time (0.10 $\pm$0.02 events in wild type cells, 0.12$\pm$0.01 events in AP2α subunit null cells, n=3 independent experiments, 34 contractile vacuoles of each cell line in each experiment were quantified). But in AP180 single and double mutant cells, the fusion frequency increased five times to ~0.5 event per vacuole (0.45$\pm$0.02 in AP180 single null, 0.55$\pm$0.04 in AP2α/AP180 DKO, n=3 independent experiments, 34 contractile vacuoles of each cell line in each experiment were quantified) (Figure 3.5B). These data suggest that the abnormally enlarged contractile vacuoles in cells lacking AP180 are the result of an increase in fusion events among their contractile vacuoles. However, the cause of the enlarged contractile vacuoles in AP2α subunit null cells remains unclear.

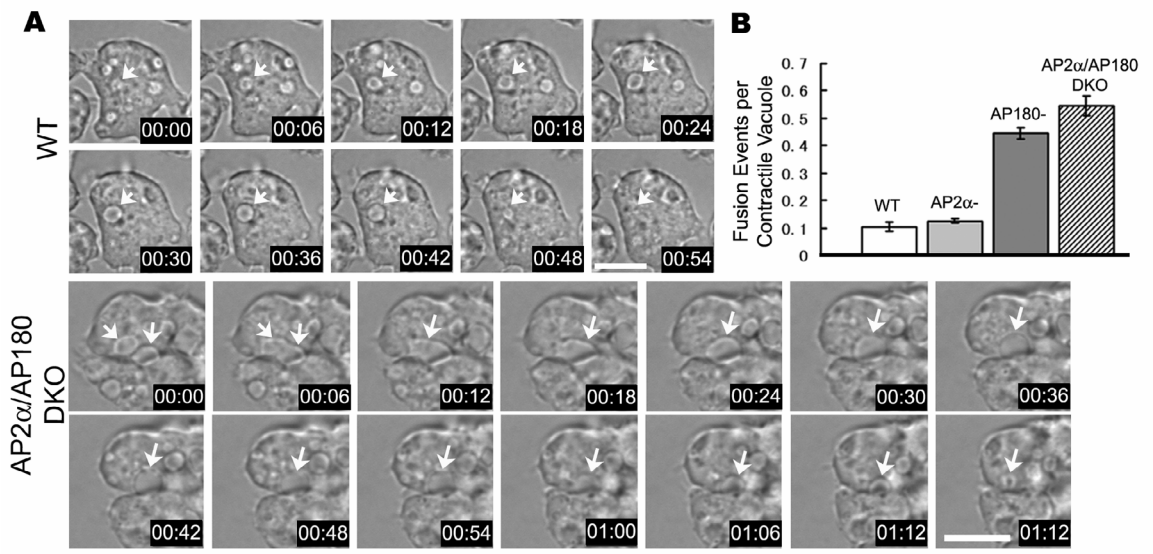


Figure 3.5 Contractile vacuoles fused with each other more frequently in the absence of AP180. (A) Time lapse of living wild type cells and AP2α/AP180 DKO cells in water. In wild type cells (WT), a whole life cycle of one contractile vacuole (arrow) is shown, from expansion to contraction. In AP2α/AP180 DKO cells (AP2α/AP180 DKO) two contractile vacuoles (arrows) fuse into a single contractile vacuole (arrow). Scale bar, 10μm. See supplemental video1.mov and video2.mov for the corresponding time lapse movies. (B) Quantification of the homotypic fusion rates of contractile vacuoles in wild type, AP2α-, AP180- and AP2α/AP180 DKO cells. Error bar, standard error, n=3 independent experiments, 34 contractile vacuoles for each cell lines were quantified in each experiment.

3.2.6 Loss of AP180 leads to an increase in Vamp7B on contractile vacuoles

I postulated that the increase in fusion among contractile vacuoles in AP180 mutants could be caused by a defect in SNARE protein traffic. Soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) are groups of transmembrane proteins that drive membrane fusion events in many organelles

(O'Halloran and Anderson, 1992b; Lefkir *et al.*, 2003). Previous studies have linked AP180 to traffic of the v-SNARE, synaptobrevin. In both *Drosophila* and *C.elegans*, AP180 is required for recycling synaptobrevin back to synaptic vesicles from the plasma membrane (Wang *et al.*, 2003; Stavrou and O'Halloran, 2006). CALM, the AP180 non-neuron homologue is also important for the endocytosis of synaptobrevin 2 (Vamp2) in cultured mammalian cells (Chen and Scheller, 2001). I therefore tested whether the loss of AP180 led to a defect in the traffic of a synaptobrevin-like protein on the contractile vacuole.

Kevin Bersuker in Dr. Arturo De Lozanne lab did a BLAST search of the *Dictyostelium* genome with the *C. elegans* synaptobrevin sequence which revealed that the closest related protein was *Dictyostelium* Vamp7B (DDB_G0277173). Then Kevin Bersuker also cloned Vamp7B into a GFP expressing vector. Expression of GFP-tagged Vamp7B in wild type cells revealed that this protein is localized on postlysosomes, secretory vesicles of the late endosomal pathway (Figure 3.6A). In addition, GFP-Vamp7B was also found on the contractile vacuole where it colocalized with clathrin and the vacuole marker Rh50 (Figure 3.6B). In contrast, a related SNARE protein, Vamp7A, was found only on endosomal vesicles but not on the contractile vacuole (data not shown and (Bennett *et al.*, 2008)). Thus, GFP-Vamp7B is a good candidate for a synaptobrevin-related protein that may be regulated by AP180 on the contractile vacuole.

Expression of GFP-Vamp7B in AP2 and AP180 mutant cells demonstrated that this SNARE protein sorted properly to their contractile vacuoles. However, there was an important difference in the intensity and localization of GFP-Vamp7B on the contractile vacuoles of AP180-null cells compared to that of wild type or AP2-null cells. In wild type cells, the postlysosomes (p80 positive compartments (Ravanel *et al.*, 2001; Mercanti *et al.*, 2006)) were labeled more strongly by GFP-Vamp7B than the contractile

vacuole (Figure 3.6A). The same relative intensity was observed in AP2α subunit null cells (Figure 5A). In contrast, the contractile vacuoles of cells lacking AP180 (AP180 null and AP2α/AP180 double null) were labeled by GFP-Vamp7B with almost equal intensity as the postlysosomes (Figure 3.6A). Not only was the staining more intense for contractile vacuoles in AP180 null cells, but more contractile vacuoles were labeled. In wild type cell, GFP-Vamp7B labeled 63±2% of the contractile vacuoles bladders (n=3 independent experiments. In each experiment 32-75 contractile vacuoles in each cell line were scored). Similar to wild type cells, 56% ±4% contractile vacuoles in AP2α subunit cells were labeled with GFP-Vamp7B. In contrast, AP180 single mutant and the AP2α/AP180 DKO displayed 90% of the contractile vacuoles labeled with GFP-Vamp7B (92±2% in AP180 null, 87±3% in AP2α/AP180 DKO,n=3 independent experiments. In each experiment 32-75 contractile vacuoles in each cell line were scored) (Figure 3.6C). Western blotting of the cells lines demonstrated that the different cell lines expressed similar amounts of GFP-Vamp7B (Figure 3.6D). The increase in the amount of Vamp7B on the contractile vacuole in the absence of AP180 suggests that AP180 may be important to retrieve Vamp7B from the contractile vacuole.

In addition to Vamp7B, I also examined Vti1, a second SNARE protein that also localized to the contractile vacuole. This Vtil-GFP plasmid was also constructed by Kevin Bersuker. Vtil allowed me to test whether the requirement for AP180 was specific for Vamp7B, by determining whether the localization of Vti1 required AP180 or AP2. I found that the distribution of Vti1 in the contractile vacuole of AP180 or AP2α null cells was indistinguishable from that of wild type cells (Figure 3.7). The similar distribution of Vti1 on the contractile vacuoles in wild type and mutant cells suggested that the defect for Vamp7B in AP180 null cells was specific for that SNARE and not a general deficit in SNARE-trafficking on the contractile vacuole.

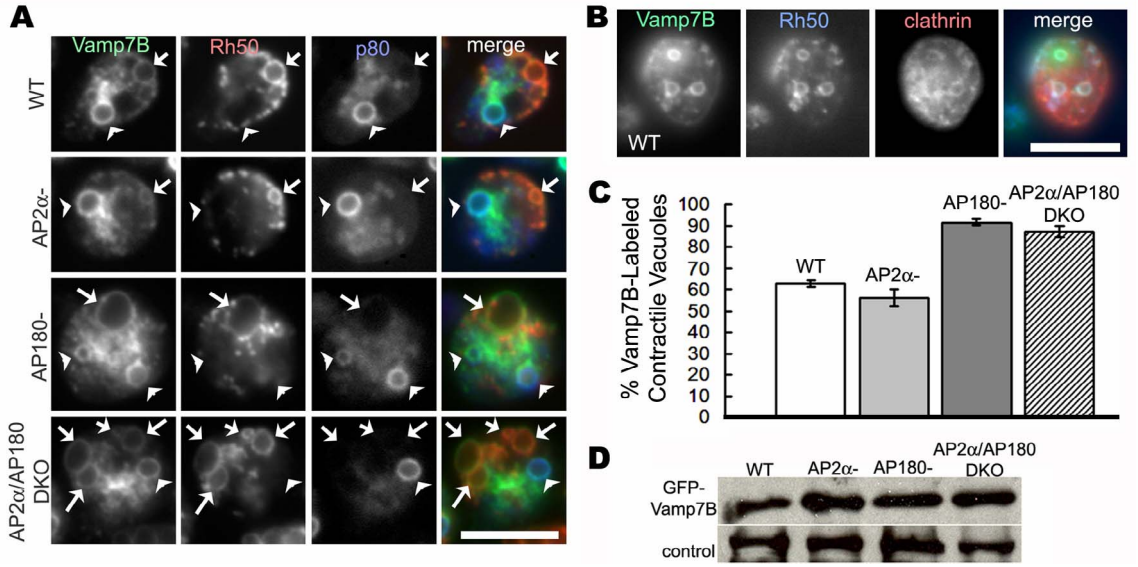


Figure 3.6 Vamp7B localizes to contractile vacuoles and postlysosomes and is enriched on the contractile vacuoles of AP180 null cells. (A) Cells expressing GFP-Vamp7B (green) were immunostained with the contractile vacuole marker, Rh50 (red) and the endosomal marker, p80 (blue). Scale bar, 10μm. (B) Vamp7B-containing contractile vacuoles label with clathrin. Wild type cells expression GFP-Vamp7B (green) were immunostained with anti clathrin light chain (clathrin) (red) and with a contractile vacuole marker, Rh50 (blue). Scale bar, 10μm. (C) Quantification of Vamp7B-labled contractile vacuoles in wild type, AP2α-, AP180- and AP2α/AP180 DKO cells. Error bar, standard error, n=3 independent experiments. In each experiment 32-75 contractile vacuoles in each cell line were scored. (D) Western blot showed an equivalent expression levels of GFP-Vamp7B. In each lane, whole cell lysates ($1x10^6$ cells) were stained with anti-GFP antibody. Anti-myosin-heavy-chain (MHC) antibody was used as a loading control.

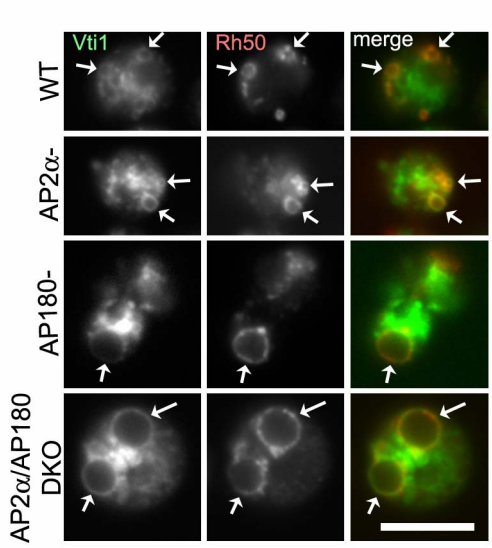


Figure 3.7 The SNARE protein Vti1 localizes equivalently on the contractile vacuoles of wild type and mutant cells. Wild type, AP2α-, AP180- and AP2α/AP180 DKO cells expressing GFP-Vti1 (green) were immunostained with the contractile vacuole marker Rh50 (red). In all four cell lines, GFP-Vti1 labels all the contractile vacuole(arrows). Scale bar, 10μm.

My results suggested that AP180 may participate in the trafficking of Vamp7B perhaps by retrieving Vamp7B from the contractile vacuole. To test whether AP180 interacted physically with Vamp7B, I performed a pull down assay. I used the cytosolic domain of Vamp7B(1-558aa) fused to GST as a bait to pull down interacting proteins from *Dictyostelium* cell lysates. For comparison purposes I used lysates from cells expressing GFP-AP180 or GFP alone as a control. As expected, GST-Vamp7B (1-558aa) were not able to pull down the control GFP alone (Figure 3.8B). In contrast, I found that GFP-AP180 was specifically pulled down by GST-Vamp7B (1-558aa) but not by GST alone, confirming a physical interaction between the two proteins (Figure 3.8A). This interaction with Vamp7B is unique to AP180 since the other two clathrin adaptor

proteins epsin and AP2 were not pulled down by GST-Vamp7B in the same assay(Figure 3.8B).

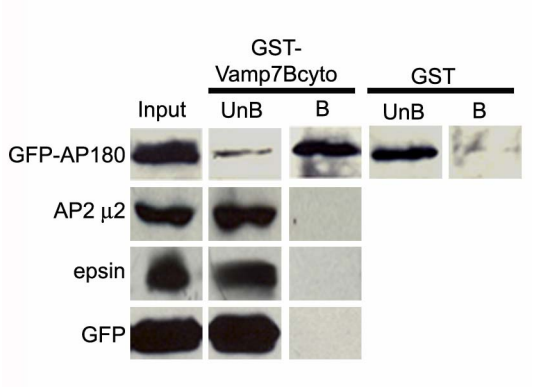


Figure 3.8 Interaction of AP180 with the Vamp7B cytosolic domain. Either the purified GST-Vamp7B cytosolic domain (GST-Vamp7Bctyo) protein or GST (negative control) was incubated with lysates of wild type cells expressing GFP-AP180. The whole cell lysate (Input) and fractions that did not bind (Unbound; UnB) or that did bind (Bound; B) to the glutathione beads were immunoblotted for GFP-AP180, for AP2 μ2 subunit, or epsin. To exclude a nonspecific interaction of the GFP tag with the GST-Vamp7B cytosolic domain, the purified GST-Vamp7B cytosolic domain was also incubated with wild type cells expressing GFP only cell lysate (GFP).

3.2.7 Characteristics of AP2α subunit null, AP180 null, epsin null, AP2α/AP180 double null, AP2α/epsin double null cell line at the plasma membrane

3.2.7.1 AP180 is still associated with plasma membrane clathrin in the absence of AP2, vice versa; AP180 helps stabilize plasma membrane AP2

In *Dictyostelium* cells, clathrin and AP2 assemble into puncta on the plasma membrane in epsin null cells and epsin does not require AP2 to associate with clathrin at the plasma membrane. Epsin formed ~20% fewer puncta on the plasma membrane of AP2α subunit null cells (Brady *et al.*, 2008). To examine whether the AP2 interacts

with AP180 in the same way as epsin, I examined the localization of AP180 in wild type and AP2α null cells expressing GFP-AP180. In both strains, GFP-AP180 localized as punctae on the plasma membrane, within the cytoplasm and in the perinuclear area. To examine whether AP180 and clathrin continued to cluster within the same punctae on the plasma membrane in AP2 mutant cells, I stained both cell lines with an antibody against clathrin light chain (Wang *et al.*, 2003). In both wild type and mutant cells, AP180 and clathrin colocalized extensively (80% of the 107 plasma membrane associated clathrin punctae in AP2α null cells, 87% of 131 plasma membrane associated clathrin punctae in wild type cells colocalized with AP180) into same punctae (Figure 3.9A).

To examine whether AP180 affects the cellular distribution of AP2, I also stained wild type DH1 cells and AP180 null cells generated in DH1 cells with the anti AP2α subunit antibody and examined the cells with fluorescence microscopy. While the total number of AP2 punctae in wild type and AP180 null cells remained similar, the distribution of AP2 punctae was different in the two cell lines. Wild type cells had 49% of 522 AP2 total punctae on their plasma membrane whereas AP180 null cells had fewer (30% of 638 total AP2 punctae) punctae on their plasma membrane (Figure 3.9B). Furthermore, staining with clathrin antibody revealed that these decrease plasma membrane AP2 punctae still colocalized with clathrin to the similar extent as in wild type cells (75% of the 108 plasma membrane clathrin punctae I quantified colocalized with AP2 in AP180 null cells, and 79% of the 88 plasma membrane clathrin punctae colocalized with AP2 in wild type cells) (Figure 3.9B).

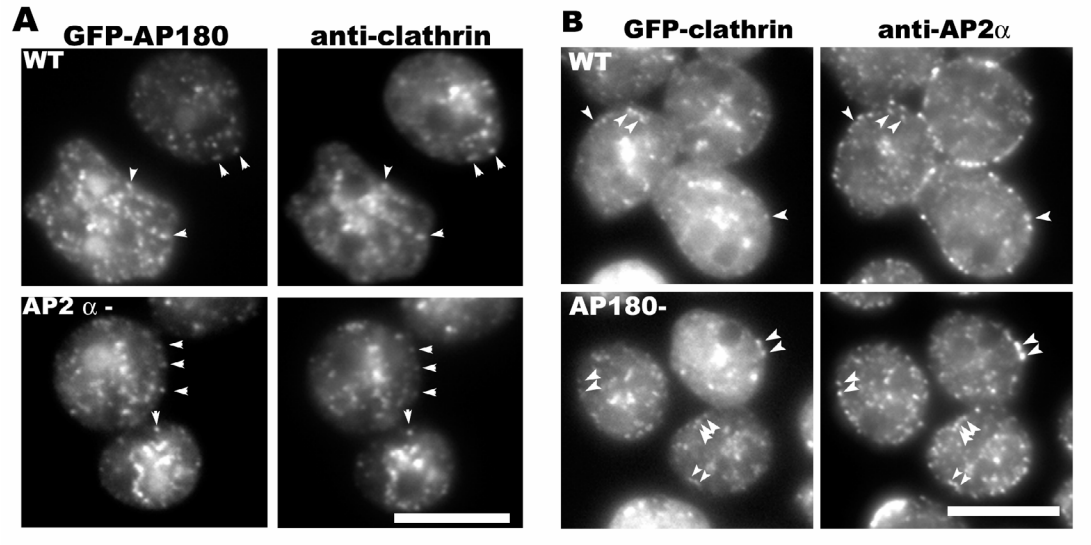


Figure 3.9 (A) AP180 does not require AP2 to localize normally and to cluster with clathrin at the same sites. Wild type and AP2α subunit null cells expressing GFP-AP180 cells were stained with clathrin light chain antibody. Arrows indicate the co-localization of AP180 and clathrin at the plasma membrane. Scale bar, 10μm. (B) Less AP2 were targeted to the plasma membrane in the absence of AP180 but AP2 still colocalized with clathrin in the absence of AP180. Wild type and AP180 null cells expressing GFP-CLC were stained with anti-AP2α subunit antibody. Scale bars, 10μm.

3.2.7.2 The plasma membrane association of clathrin is reduced in the absence of AP2 and/or AP180, the absence epsin does not affect the plasma membrane clathrin

AP2 and AP180 synergistically promote maximum clathrin assembly activity *in vitro* (Lindner and Ungewickell, 1992; Hao *et al.*, 1999). To examine how the absence of two major adaptor proteins affect the association of clathrin with the plasma membrane in living cells, I stained wild type, AP2α-, AP180-, epsin-, AP2α/AP180 DKO and AP2α/epsin DKO cell lines with an antibody against clathrin light chain, and imaged the cells using confocal microscopy. In all the cell lines, clathrin localized to punctae at

the periphery and in the cytoplasm of the cells; however in AP2α/AP180 DKO cells, fewer clathrin punctae were seen on the plasma membrane (Figure 3.10A). To quantify the decrease of the clathrin punctae at the plasma membrane, I calculated the intensity of fluorescence per area in confocal sections focused on the plasma membrane (the same method as in Chapter 2). I found 17% and 23% reduction in clathrin association with the plasma membrane in AP2 and AP180 single mutant cells respectively, while in the AP2α/AP180 DKO cells I found 31% decrease in clathrin at the plasma membrane (Figure 3.10B). Interestingly I did not observe any plasma membrane clathrin deduction in the epsin null cells. These results indicated that clathrin has lost some, but not all, of its ability to associate with the plasma membrane in the absence of both AP2 and AP180, but not epsin. More importantly, the decrease of plasma membrane associated clathrin in the AP2α/AP180 DKO (31%) is close to the simple addition of AP2 and AP180 single mutant cells (17%, 23%). Thus I conclude that *in vivo*, AP2 works additively with AP180 but not epsin to recruit clathrin onto the plasma membrane.

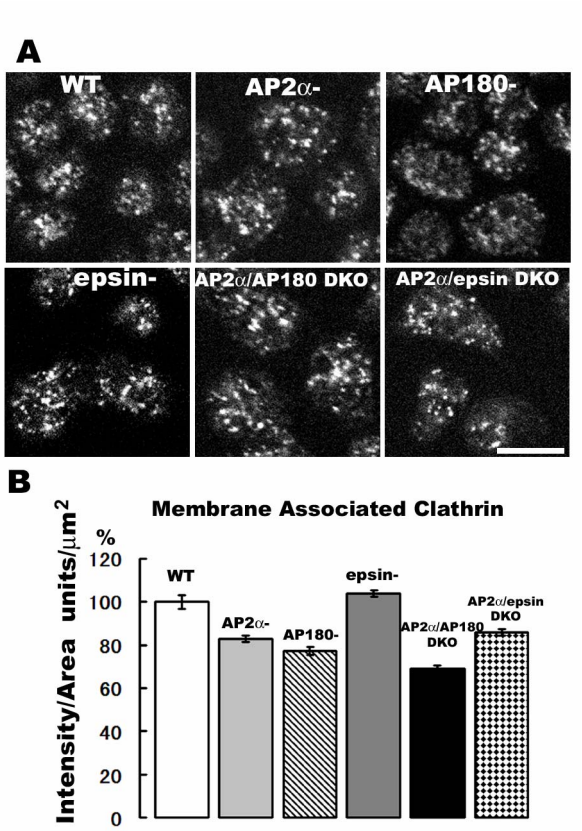


Figure 3.10 The membrane association of clathrin is reduced in AP2α subunit null and/or AP180 null cells but not in epsin single null cells. Wild type, AP2α null, AP180 null, epsin null and AP2α/AP180 double null, AP2α/epsin cells were fixed and stained with an antibody against clathrin light chain (A) The images were focused on the top of the cells in order to compare clathrin association at the plasma membrane in the different cells lines. Scale bars 10μm. 90-110 cells were quantified in each cell lines. Scale bars, 10μm. Clathrin punctae were quantified in each cell line indicated (B) The membrane association of clathrin was reduced by 17% in AP2α null cells, 23% in AP180 null cells and 31% in AP2α/AP180 double null cells. But deletion of epsin did not have an effect on clathrin on the plasma membrane. Error bar, standard error among cells within the same cell line.

3.2.8 Characteristics of AP2α subunit null, AP180 null, epsin null, AP2α/AP180 double null, AP2α/epsin double null cell line in cytokinesis and cell development

Dictyostelium discoideum cells use two distinct cytokinesis mechanisms. Cells use a myosin II-dependent cell division in suspension and an adhesion-dependent but myosin II-independent mechanism on substrates (Nagasaki *et al.*, 2001). Clathrin heavy chain mutants have a severe growth defect with multinucleated cells in suspension which links clathrin function to cytokinesis (Niswonger and O'Halloran, 1997b; Gerald *et al.*, 2001). Interestingly, similarly to clathrin heavy chain null cells, epsin null cells also accumulated multinuclei when grow in suspension media ((Brady *et al.*, 2008) and Figure 3.11 A and B). However, in suspension AP2α null, along with AP180 null and AP2α/AP180 double null cells, were mostly single nucleated like wild type cells. AP2α/epsin double null cells displayed a cytokinesis defect to the same extend as epsin single null cells. I quantified the average number of nuclei in each cell line and compared the different cell lines. When grown in suspension, wild type cells, AP2α null cell, AP180 null cells and AP2 α/AP180 DKO cells all had 2 $\pm$ 0 nuclei/cell in average. In contrast, epsin null had 5 $\pm$1 nuclei/cell, and AP2α/epsin DKO had 4 $\pm$1 nuclei/cell.

In contrast with growth in suspension, AP2α subunit null cells had a cytokinesis defects when grown on substrates (Chapter 2 and (Figure 3.11A,B)). I then quantified how many nuclei each cell had when grown on substrate. Wild type, AP180 null, epsin null all had 1 $\pm$0 nuclei/cell whereas all three AP2α mutant cell lines had 2 $\pm$0 nuclei/cell. This result suggested that AP2 works in the same myosin II-independent cytokinesis pathway whereas epsin, along with clathrin, is clearly involved in the myosin II-dependent pathway.

Dictyostelium cells exist as two phases: vegetative phase and development phase. Upon starvation, the *Dictyostelium* cells enter a developmental program in which about

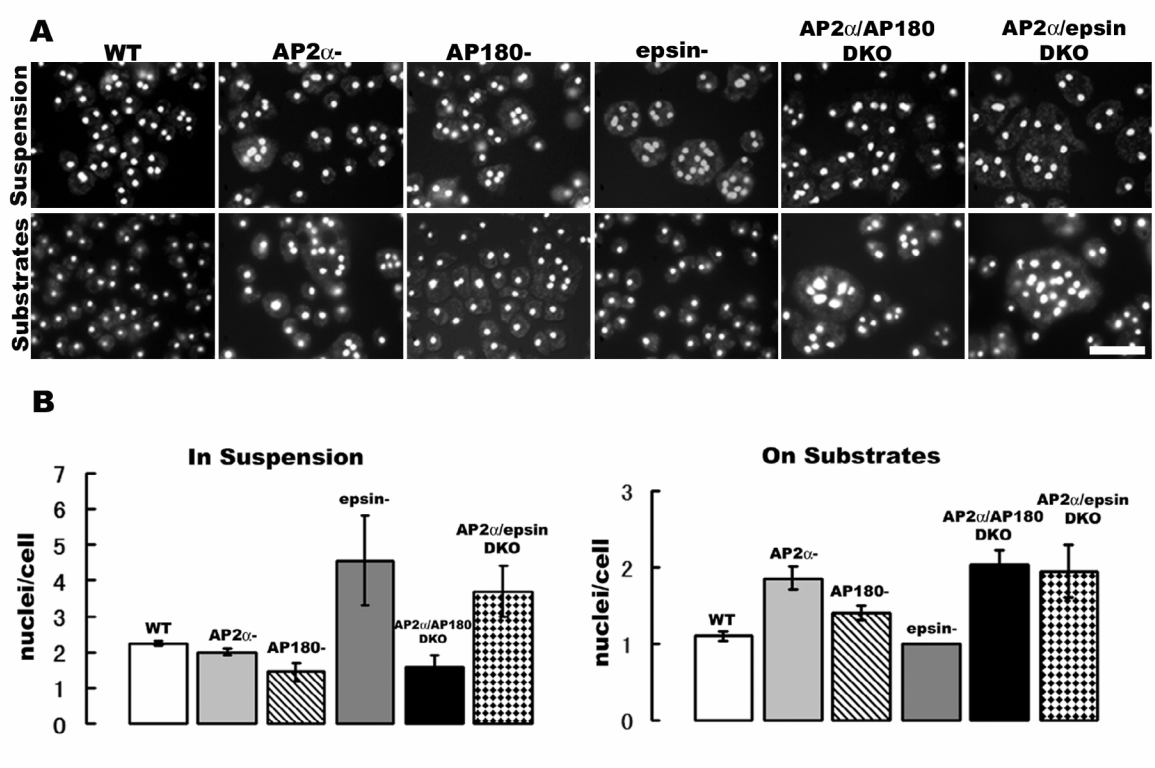
100,000 amoebae cells aggregate and differentiate, form a multicellular organism and construct a fruiting body made up by oblong spores on the top of a stalk (Kessin, 2001). Clathrin heavy chain mutant cells are not able to initiate the development cycle (O'Halloran and Anderson, 1992b). When all our six cell lines in this study were put into starvation condition, they all developed into fruiting bodies. AP2α mutant cells including the AP2α/AP180 DKO and the AP2α/epsin DKO cells made fruiting bodies but with shorter stems (Figure 3.11C top panel).

Two of the clathrin accessory proteins in *Dictyostelium*, Hip1r and epsin null cell lines develop normal looking fruiting bodies but contain abnormally round spores (Repass *et al.*, 2007) (Brady et al, 2008, submitted). In this study I also noted that the spores produced by AP2α null cells were slightly rounder (Figure 3.11C bottom panel). On the other hand, the AP180 null cells formed normal oblong spores whereas the AP2/AP180 DKO cells behaved similarly to AP2α subunit null cells. Moreover, the AP2/epsin DKO cells produced round spores similar to epsin null cells. To be able to compare the spore shape among cell lines, I measured the width:length ratio of spores (Figure 3.11D). The ratio of wild type spores was 0.65 ± 0.02 (n=42; mean ± SE); spores derived from epsin null mutants had a ratio of 0.84 ± 0.01 (n=45; mean ± SE). AP2α mutant spores were slightly round with the number of 0.79 ±0.02 (n=38; mean ± SE) whereas AP180 mutant spores were wild type shape with the number of 0.65 ±0.01 (n=40; mean ± SE). However, I did not observe an additive effect of AP2 and epsin in the AP2α/epsin DKO spore: the ratio of this AP2α/epsin DKO spores was very close to the epsin single mutant spores with a ratio of 0.86±0.02 (n=40; mean ± SE).

Since the plasma membrane localization of epsin is important for its function to maintain normal spore shape (Brady, personal communication) and the absence of AP2

abolished about 20% of the plasma membrane associated epsin, I conclude here that AP2 affects the spore morphology through mislocalizing epsin.

The short stem phenotype I observed in the DH1 AP2α subunit null cells is different from the AP2α null mutant cells in Ax2 background as I demonstrated in Chapter 2. AP2α null cells generated in Ax2 wild type cells make normal height stems with smaller fruiting bodies during development. I think this conflicting result was caused by cell line differences between Ax2 and DH1 cell line.



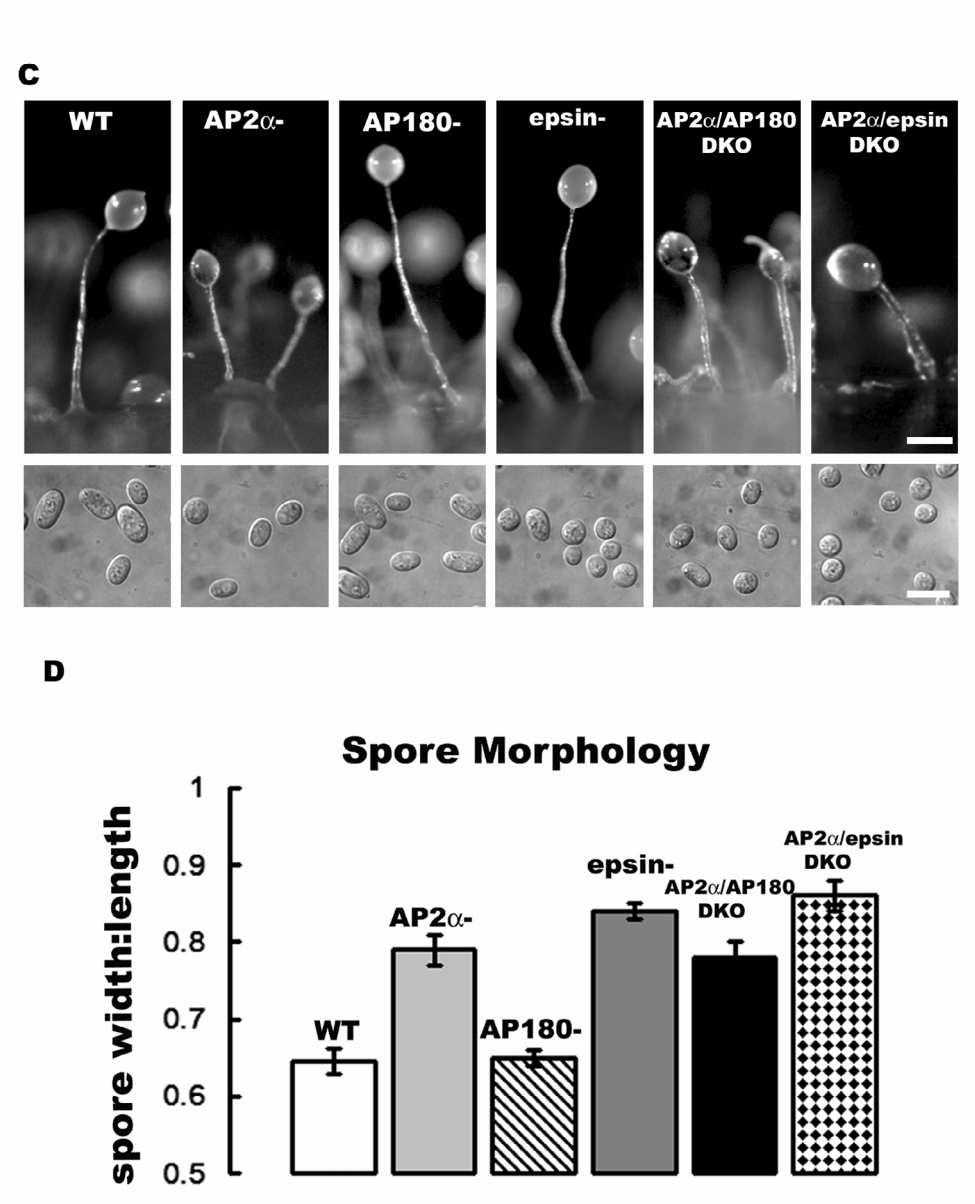


Figure 3.11 Characterizing different *Dictyostelium* mutants in cytokinesis and cell development. (A) Cells grown either attached to Petri dish or in suspension medium for 72 hours were stained with DAPI for nuclei. Scale bars 50μm. (B) Nuclei per cell were quantified in all six different cell lines grown on substrates or in suspension medium. 220-450 cells were quantified in each cell line for three independent experiments. Error bar, standard error of three independent experiments. (C) Upper panel shows that AP2α

subunit mutant cell lines make fruiting bodies with short stems. *Dictyostelium* cells were starved on the starvation plates to induce cell development and visualized after 24 hours. Scale bars 100μm. Lower panel shows both AP2α null and epsin null cells had rounder spores. Spores from developed *Dictyostelium* cells fruiting bodies were collected and visualized. Scale bars 10μm. (D) Quantification of the spore morphology by calculating the ratio of spores width: length indicates that epsin null cells and the AP2 α/epsin double null cells have the roundest spores among all the cell lines. AP2α null cell lines, including AP2α/AP180 double null cells, had slightly round spores. 38~45 spores were quantified for each cell lines. Error bar, standard error among different spores within the same cell lines.

3.3 DISCUSSION

In this Chapter, I first showed that contractile vacuole associated clathrin coated vesicles share several clathrin adaptors in common with the ones on the plasma membrane, one of which, AP180, regulates the amount of the Vamp7B SNARE at the contractile vacuole. Of all clathrin adaptor proteins described in *Dictyostelium* AP180, AP2 and epsin were found on both plasma membrane and contractile vacuoles, while Hip1r was found only on the plasma membrane. It is clear that clathrin coated vesicles budding from the contractile vacuole are important for the function of this organelle, as mutations in AP180 and AP2 produce abnormal vacuoles. In addition, using both GST pull down and immunostaining, I identified AP180 as the possible adaptor protein for Vamp7B trafficking in clathrin coated vesicles. Furthermore, I disclosed the mechanism of the abnormally large contractile vacuoles in AP180 null cells: excessive homotypic fusion among contractile vacuoles. Recycling Vamp7B from contractile vacuole through clathrin coated vesicles is an efficient way to prevent those excessive fusions.

In this Chapter, I have also shown that AP2 works additively with AP180 in recruiting ~31% of plasma membrane associated clathrin in DH1 cell line background. In addition, AP2, AP180 and epsin behave differently in clathrin related cytokinesis, cell development.

3.3.1 AP180 serves as a clathrin adaptor protein that retrieves Vamp7B from contractile vacuoles

Previous studies have shown that AP180 is required for the proper localization of synaptobrevin in metazoans (Nonet *et al.*, 1999; Bao *et al.*, 2005; Harel *et al.*, 2008). I

demonstrated that AP180 is required for retrieval of the synaptobrevin homologue, Vamp7B from *Dictyostelium* contractile vacuoles. Loss of AP180 caused an increase in the labeling of contractile vacuoles with Vamp7B. Concomitant with the increase in Vamp7B, I observed an increase in the homotypic fusion among contractile vacuoles leading to the formation of enlarged vacuoles in AP180 mutant cells. This role of AP180 is specific for Vamp7B, since another contractile vacuole-associated SNARE protein, Vtil, was not affected in AP180 mutant cells. In addition, the GST-Vamp7B cytosolic domain pulled down AP180 but not other contractile vacuole associated AP2 and epsin from cell lysates, indicating a physical interaction between AP180 and Vamp7B. It is known that SNARE proteins lack the known linear peptide binding-motifs for clathrin adaptors. However, recent studies have revealed the possible mechanism by which clathrin adaptors bind to SNARE proteins. A surface-to-surface interaction between SNARE protein Vtilb and epsinR has been suggested by crystallography (Miller *et al.*, 2007). In addition, the mammalian clathrin adaptor protein Hrb directly mediates the internalization of Vamp7 through the adaptor protein's unstructured tail domain (Pryor *et al.*, 2008). My results provide evidence that AP180 also serves as adaptor to retrieve SNARE protein Vamp7B in clathrin coated vesicles. Since the identified association between Vamp7 and Hrb, EpsinR and Vtilb are believed to be unique for the specific SNARE adaptor protein combination, how exactly AP180 associates with Vamp7B in *Dictyostelium* cells needs to be further determined.

In this study, I showed that in wild type cells, Vamp7B labels ~60% of contractile vacuoles. How is Vamp7B delivered to the contractile vacuole and where does Vamp7B go once it is retrieved from the contractile vacuole? Mammalian Vamp7 is required for the heterotypic fusion between late endosomes and lysosomes and also during the fusion between lysosomes and plasma membrane (reviewed by (Luzio *et al.*,

2007)). I observed that *Dictyostelium* Vamp7B is found not only on contractile vacuoles but also on vesicles of the endocytic pathway (the postlysosome) where it probably plays a role similar to mammalian Vamp7B. I propose that Vamp7B has an additional role during the heterotypic fusion of vesicles transporting components to the contractile vacuole. Among these are Golgi-derived vesicles with newly synthesized proteins (Schneider *et al.*, 2000; Gerisch *et al.*, 2004) and plasma membrane-derived vesicles that recycle contractile vacuole proteins (Mercanti *et al.*, 2006). After those vesicles fuse with contractile vacuoles, Vamp7B molecules are left on the contractile vacuoles where NSF may then break apart the resulting SNARE complexes. Once on contractile vacuoles, Vamp7B may participate in a normal basal level of homotypic fusion among contractile vacuoles. Many groups have described the dynamic behavior of contractile vacuole networks including homotypic fusion (Clarke *et al.*, 2002). However, an increase in the amount of Vamp7B on contractile vacuoles would lead to an increase in fusion rates and the formation of abnormally large contractile vacuoles. To prevent this situation, AP180 recruits Vamp7B into clathrin coated vesicles to recycle the SNARE to its originating compartment, probably endocytic compartments. Therefore, clathrin fulfills one of its tasks on contractile vacuoles: recycle one important SNARE proteins through AP180. Considering the much more severe contractile phenotype of clathrin heavy chain mutant cells, I believe that there are more uncovered roles of clathrin coated vesicles on contractile vacuoles.

3.3.2 The role of AP2, epsin and Hip1r in contractile vacuole function

I demonstrated that the adaptor protein AP2 also plays an important role in the regulation of contractile vacuole size, but the localization of Vamp7B was not altered on the contractile vacuoles of AP2α subunit null cells and GST-Vamp7B was not able to

pull down AP2 from whole cell lysate. Accordingly, the contractile vacuoles of AP2α subunit null cells did not fuse abnormally. In addition, the contractile vacuoles of AP2α subunit null cells were enlarged only in a hypotonic environment whereas the vacuoles of AP180 null cells were large in both isotonic and hypotonic environments. Significantly, the contractile vacuoles of the double mutant cells were significantly larger than those of the single mutant cells, indicating a cumulative effect. These differences between AP2α subunit null and AP180 null cells suggest that AP2 has a different role in the contractile vacuole than AP180. One possibility is that AP2 regulates v-ATPase proton pumps.

The v-ATPase proton pump is universally found on acidic vesicles of the endolysosomal pathway but is also found on other organelles. The v-ATPase is also a major component of contractile vacuole membranes, found both on the bladder and the tubule network (Bush *et al.*, 1994). The v-ATPase is important to drive water accumulation from the cytosol into the contractile vacuoles (Clarke *et al.*, 2002). Interestingly, it has been shown that the v-ATPase is present at the plasma membrane in association with clathrin (Marquez-Sterling *et al.*, 1991), and that the AP2 μ2 subunit, the cargo-binding subunit of AP2, binds to v-ATPase *in vitro* (Myers and Forgac, 1993; Liu *et al.*, 1994). Thus, it is possible that on *Dictyostelium* contractile vacuoles, AP2 is involved in recycling certain amount v-ATPase from the contractile vacuole membrane through clathrin coated vesicles to other organelles, such as endosomes. When AP2 is missing, v-ATPase proteins may accumulate on the contractile vacuole resulting in an increase in water transport into the bladder, but only when the cells are exposed to a hypotonic environment. This model of AP2 function needs to be tested by developing tools to study the rate of water transport into the vacuoles of AP2 null cells.

It has been reported that AP2 and AP3 regulate the localization of another *Dictyostelium* synaptobrevin homologue, Vamp7A (Bennett *et al.*, 2008). Since I found

that AP2 does not control the localization of Vamp7B, my results suggest that two Vamp7 homologues in *Dictyostelium* are sorted through different clathrin adaptors into clathrin coated vesicles.

The clathrin accessory protein epsin is the third protein I observed on contractile vacuoles. Unlike AP2 and AP180, I did not find any contractile vacuole related phenotype in epsin null cells. On the plasma membrane epsin inserts its ENTH domain into the plasma membrane bilayer and induces the membrane curvature and clathrin coated pit invagination. In *Dictyostelium* cells, the localization of clathrin on the plasma membrane and clathrin-related fluid phase uptake were not affected in epsin null cells which suggests a limited role of *Dictyostelium* epsin on plasma membrane clathrin coated vesicle invagination (Brady *et al.*, 2008). On the contractile vacuole, epsin may also play a non-essential role in promoting membrane curvature to facilitate the clathrin coated vesicle formation just like it does on plasma membrane. This could explain why we observed epsin on contractile vacuoles, but we did not find any contractile vacuole related phenotypes in epsin null cells.

Actin filaments play a very important role during clathrin coated vesicle scission. Clathrin coated vesicles internalization is inhibited by addition of latrunculin (Kaksonen *et al.*, 2003). Clathrin accessory protein Hip1r links the clathrin coated vesicles with cortical actin filaments during endocytosis (McCann and Craig, 1997; Yang *et al.*, 1999; Brett *et al.*, 2006). But unlike on the plasma membrane, I did not detect Hip1r on the contractile vacuoles membranes. This leads to the possibility that the scission of clathrin coated vesicle from the contractile vacuole membrane is not actin dependent.

3.3.3 The contractile vacuole, a novel system for studying clathrin mediated traffic

The contractile vacuole offers a unique system for the study of how clathrin coated vesicles can remodel the membrane of a particular organelle. In addition to the plasma membrane, clathrin coated vesicles have been observed in multiple internal organelles including the TGN, endosomes, and lysosomes. In addition to *Dictyostelium* cells, clathrin coated vesicles have been found on the contractile vacuoles of a wide variety of protists including the alga *Vacuolaria virescens* (Heywood, 1978; Patterson, 1980)**.** Our identification of a population of functional clathrin coated vesicles with multiple clathrin accessory proteins on the *Dictyostelium* contractile vacuoles offers an experimental system to dissect the contribution of individual proteins of coated pits to contractile vacuoles in a genetically tractable system.

3.3.4 *Dictyostelium* cell line difference in contractile vacuole sizes

In contrast with my results, Stavrou *et al*. reported a different result in 2006. When she examined AP180 null cells created from Ax2 wild type *Dictyostelium* cells background, she concluded that there is a 43% increase of clathrin-associated contractile vacuoles (47% in wild type Ax2 cells, 90% in AP180 null) (Stavrou and O'Halloran, 2006). To explain the discrepancy, I first compared the contractile vacuole sizes in the cells lines from different background. In Ax2 wild type background, the difference between contractile vacuoles diameters at their maximum sizes in wild type cells and AP180 nulls is around 1.7 μm. So even though the increase of the association between clathrin and contractile vacuoles may reflect an increased time for clathrin cage assembly in the absence of AP180, it is also possibly caused by the increased size of contractile vacuoles in the mutant cells as well. Moreover, this factor may totally compensate for the loss of clathrin on contractile vacuoles caused by the absence of AP180. However,

in the DH1 wild type background, the contractile vacuole size difference between wild type cells and AP180 null cells is only 0.7μm in diameter, much smaller than in the Ax2 background. Therefore in this study, I observed a loss of clathrin in the contractile vacuoles when I deleted AP180 in the DH1 background.

Chapter 4 Conclusions and Future Direction

AP2 is considered an essential adaptor protein during clathrin mediated endocytosis in multicellular systems, but AP2 is not required for normal clathrin function in yeast. Thus the contribution AP2 to clathrin function remains inconclusive. In Chapter 2, I reported that I identified the AP2α subunit and assessed an α subunit null mutant strain in the unicellular organism, the social ameba *Dictyostelium discoideum*. I concluded that AP2 is important, but not essential, for *Dictyostelium* cells: it is required for recruiting a percentage of the total clathrin assembled on the plasma membrane and AP2 also contributes to several clathrin related cellular processes including cytokinesis and the cell developmental cycle.

In addition to the plasma membrane localization, clathrin coated vesicles are found on some internal organelles, including *Dictyostelium* contractile vacuoles. But the composition and function of these contractile vacuole associated clathrin coated vesicles is not clear. In the second part of my Ph.D study (Chapter 3), I studied the role of clathrin and its adaptor proteins to the organization and function of the contractile vacuole complex. I concluded that clathrin coated vesicles on contractile vacuoles contain clathrin adaptor proteins AP2, AP180 and epsin but not Hip1r. I found that AP180 could serve as an adaptor protein to traffic the v-SNARE protein Vamp7B away from the vacuole in clathrin coated vesicles. Thus AP180 prevents excessive homotypic fusion among contractile vacuoles. To a lesser degree, AP2 is required to limit contractile vacuole size, but the mechanism is unknown. On the other hand, I did not find a role for epsin in contributing to contractile vacuole organization or function.

4.1 POSSIBLE EVOLUTION PATH OF AP2 IN CLATHRIN MEDIATED ENDOCYTOSIS

Clathrin adaptor proteins bind both clathrin and the plasma membrane simultaneously. This group of proteins includes AP family members, CALM/AP180 and epsin. Even though these clathrin adaptor proteins do not share prominent sequence similarities, they all share a common structural design: the large subunits of AP family members, CALM/AP180 and epsin all have distinct folded domain(s) connected to a long flexible linker (Evans and Owen, 2002). These folded domains bind to cargo, membranes and other adaptor proteins while the linkers have binding motifs for clathrin. This structural design allows these adaptor proteins to link clathrin to the plasma membrane and establish a complex dynamic network (Schmid and McMahon, 2007). The similarity in structure also provides the potential for clathrin adaptor proteins to be functionally redundant when it is necessary and this may explain why in single cell organism such as yeast and *Dictyostelium*, AP2 and/or AP180 are not required for clathrin related cellular pathways including clathrin localization (Gonzalez-Gaitan and Jackle, 1997; Grant and Hirsh, 1999; Huang *et al.*, 1999; Yeung *et al.*, 1999; Mitsunari *et al.*, 2005). In unicellular organisms, other clathrin adaptor proteins, such as epsin and/or AP180 may share functional redundancy with AP2 and they all traffic their own specific cargo and recruit clathrin onto the plasma membrane in their own rights. When AP2 is missing, other adaptor proteins with similar structural design may compensate for the loss of AP2 to some extent. Through evolution, it is possible that individual clathrin adaptor proteins gained their unique roles during endocytosis: AP2 became the most important adaptor protein to recognize cargo and recruit clathrin onto the plasma membrane; AP180 became specialized in regulating clathrin coated vesicle size and epsin functions specifically in membrane invagination. Since these clathrin adaptors do not share sequence similarity, it is likely that it is a parallel evolution of similar domains.

Because of the greater functional specificity, in complex systems, AP2 mutants my display very severe phenotypes and clathrin adaptor proteins are not functionally interchangeable with AP2.

4.2 VAMP7B IN CONTRACTILE VACUOLES

4.2.1 Vamp7B mediates contractile vacuole homotypic fusion

In Chapter 3, I proposed that loss of AP180 resulted in an excessive amount of Vamp7B on the contractile vacuole. This excessive Vamp7B induces an increase in homotypic fusion among contractile vacuoles, resulting in the abnormally large contractile vacuoles I observed in AP180 null cell lines. However, whether Vamp7B can induce contractile vacuole homotypic fusion has not been directly tested. One experiment to examine whether Vamp7B can directly induce contractile vacuole fusion is to assess the contractile vacuole fusion rate in cells over-expressing Vamp7B. I predict that wild type cells over-expressing Vamp7B would exhibit an increased fusion rate of contractile vacuoles homotypic fusion. But when I assessed the contractile vacuole size and fusion rates in wild type cells over-expressing GFP-Vamp7B, I failed to observe any increase of the contractile vacuole size or homotypic fusion rate (data not shown). This seemingly conflicting result can be explained by several possibilities. The first possible reason is that since membrane fusion is initiated the direct association of SNAREs from the two membranes; Vamp7B may need an equal amount of a second contractile vacuole associated SNARE protein to accomplish the contractile vacuole homotypic fusion. However, in wild type cells with over-expressed Vamp7B, the increased level of Vamp7B may not be accompanied by an increase in its SNARE partner. This wild type level of this Vamp7B partner could be the limiting factor to induce more fusion in those

over-expressed cells. If this is the reason, then why in AP180 null cells, does an increased number of contractile vacuole associated Vamp7B cause more fusion? This can be explained if AP180 traffics the SNARE partner of Vamp7B, as well as Vamp7B. If this is the case, in AP180 null cells, both Vamp7B and its SNARE partner would be enriched on contractile vacuoles. The second possibility to explain the normal contractile vacuole fusion rate in wild type cells over-expressing GFP-Vamp7B is that the GFP tag on the N-terminal, also the cytoplasmic terminal, of Vamp7B interferes with Vamp7B normal function to intertwine with its partner during fusion. GFP tagged Vamp7B may be sorted properly, but it may not be fully functional. To address this possibility, we will need to test whether GFP-Vamp7B can fully rescue Vamp7B mutant phenotypes once the Vamp7B mutant cell line is available.

Another way to elucidate whether Vamp7B can initiate homotypic fusion is to examine the contractile vacuole fusion in Vamp7B-depleted cells. Since Vamp7B is involved in the fusion among endocytic compartments, deleting the Vamp7B gene could be lethal to *Dictyostelium* cells. Therefore measuring the contractile vacuole fusion rates in Vamp7B RNAi knockdown cell line with reduced Vamp7B level may be able to answer this question. Generating Vamp7B RNAi knockdown cell line is in progress in Dr. Arturo De Lozanne's lab. However, since the contractile vacuole fusion rate in wild type cells is already very low (0.1 event/contractile vacuole), it could be difficult to detect a possible decrease in the fusion rate in the Vamp7B null cells.

4.2.2 The partner of Vamp7B on the contractile vacuoles

If Vamp7B can directly induce the homotypic fusion among contractile vacuoles, which SNARE protein is the partner of Vamp7B? One candidate is the t-SNARE protein Vti1. A previous study has identified *Dictyostelium* v-SNARE Vamp7A and t-

SNARE Vti1 in the same SNARE complex (Bogdanovic *et al.*, 2002). In addition, I also observed a contractile vacuole localization of Vti1 in *Dictyostelium* wild type cells (Figure 3.7). Is Vtil the partner for Vamp7B on the contractile vacuoles? One way to answer this question is to examine whether there is a direct interaction between Vamp7B and Vti1 using *in vitro* binding assay. Since both Vamp7B and Vti1 are transmembrane proteins, their cytosolic domains would be purified and used for this assay and tethering factors should also be added in this assay.

If Vtil is the partner for Vamp7B on the contractile vacuoles, the next question is which clathrin adaptor protein mediates Vti1 trafficking? Even though I did not observe a mislocalization of Vti1 in the absence of AP180, it is still possible that Vtil is transported by AP180. Since Vti1 is abundant on the contractile vacuoles (100% of the contractile vacuoles are strongly labeled with GFP-Vti1), the amount of mislocalized Vtil caused by the absence of AP180 may not be detected with immunofluorescence microscopy. A GST pull down assay to examine whether Vtil has a direct interaction with AP180 could evaluate this possibility.

4.3 REVEAL THE MECHANISM OF AP180 AND VAMP7B INTERACTION

Surface to surface interactions between SNARE protein Vtilb N-terminal H_{abc} domain and the epsinR ENTH domain have been suggested by crystallography (Miller *et al.*, 2007). In addition, the mammalian clathrin adaptor protein Hrb mediates the trafficking of the Vamp7 by binding its unstructured C-terminal 20 residues to the Vamp7 N-terminal longin domain (Pryor *et al.*, 2008). However the binding modes between Vamp7/Hrb and epsinR/Vtilb are believed to be unique for the specific SNARE adaptor protein combination. Thus how AP180 associates with *Dictyostelium* Vamp7B needs further investigation.

To understand the interaction between AP180 and Vamp7B, it is important to investigate which domains contribute to the binding between these two proteins. Epsin ENTH domain and AP180 ANTH domain are two plasma membrane binding domains with similar structures (Ford *et al.*, 2002). Since the ENTH domain of epsinR directly binds Vti1, it is possible that the AP180 ANTH domain similarly binds the SNARE protein Vamp7B. An *in vitro* binding assay using purified AP180 ANTH domain and Vamp7B cytosolic domain would help to elucidate this possibility. However, since the binding mode between epsinR and Vti1b is probably unique, other domains of AP180 could interact with Vamp7B.

To thoroughly understand the interaction between AP180 and Vamp7B it is also important to reveal which domain of Vamp7B contributes to the AP180/Vamp7B binding. The longin domain of mammalian Vamp7 is responsible for the binding between the SNARE protein and clathrin adaptor protein Hrb. Therefore the N-terminal longin domain of Vamp7B could be the domain that binds to AP180. *In vitro* binding assays using purified Vamp7B longin domain and purified AP180 could be used to examine whether Vamp7B longin domain has the ability to interact with AP180 directly. Once the binding sites on both AP180 and Vamp7B for this SNARE protein/clathrin adaptor complex are determined, crystallography can help to reveal the exact binding mode and further help to understand the relationship between SNARE proteins and clathrin adaptor proteins.

AP180 has been considered as a non-cargo binding protein although an earlier study has linked its function to the endocytosis of EGF receptor in HeLa cells (Huang *et al.*, 2004). Understanding the interaction between AP180 and Vamp7B will also help to re-evaluate AP180 as a possible cargo binding adaptor protein during clathrin mediated pathways.

4.4 CLATHRIN COATED VESICLES ARE REQUIRED FOR CONTRACTILE VACUOLES RESIDENT PROTEINS TRAFFICKING

Clathrin coated vesicles have been observed on *Dictyostelium* cells in multiple studies (Heuser, 2006; Stavrou and O'Halloran, 2006). However, the exact function of clathrin coated vesicles on the contractile vacuole is not very well understood. In this study, I proposed that clathrin coated vesicle recycles Vamp7B away from the contractile vacuole in an AP180 dependant fashion. However, it is believed that clathrin may facilitate the trafficking of other contractile vacuole related proteins. In support of that, it has been shown that, unlike the enlarged contractile vacuoles in AP180 null cells, clathrin heavy chain null cells do not have organized contractile vacuole complexes. Instead, these cells have only dispersed small vacuoles, which indicates a critical role of clathrin in contractile vacuole organization (O'Halloran and Anderson, 1992b; Stavrou and O'Halloran, 2006). In addition, in clathrin adaptor protein AP1 mutant cells, several contractile vacuole markers are mislocalized (Lefkir *et al.*, 2003) which suggests that clathrin mediated trafficking is required for transporting resident proteins to the contractile vacuole. Moreover, Heuser also observed that after contractile vacuoles collapse at the plasma membrane, clathrin coated vesicles can re-internalize important contractile vacuole components to regenerate contractile vacuoles (Heuser, 2006). Together, these data strongly suggest an important role of clathrin in contractile vacuole biogenesis. To understand how clathrin contributes to contractile vacuole function, more contractile vacuole associated cargos of clathrin mediated trafficking need to be identified. Vti1 and v-ATPase are two possible candidate cargos for clathrin mediated trafficking. It has been shown that Vti1 and v-ATPase proton pumps are two *Dictyostelium* contractile vacuole resident proteins and are also associated with clathrin coated vesicle components in mammalian cells: Vti1 binds epsinR and v-ATPase can

bind AP2 complex μ2 subunit (Marquez-Sterling *et al.*, 1991; Myers and Forgac, 1993; Liu *et al.*, 1994; Miller *et al.*, 2007). The function of Vti1 on contractile vacuole is still unknown, but it is known to promote fusion at endosomal membranes and trans-Golgi network, therefore it could be an important SNARE protein that promotes fusion during the formation of contractile vacuoles (Pryor *et al.*, 2004; Murray *et al.*, 2005). *Dictyostelium* v-ATPase is required for contractile vacuoles function: it drives water from the cytosol into the contractile vacuoles (Clarke *et al.*, 2002). Therefore further studies to test whether clathrin and its adaptors mediate the trafficking of Vti1 and v-ATPase will help to understand more about the function of clathrin coated vesicles in contractile vacuole biogenesis.

Chapter 5 Experimental Procedures

5.1 MATERIAL AND METHODS

5.1.1 Electroporation

Plasmids were transformed into wild type and mutant cells by electroporation. $5x10^6$ cells were washed twice with ice-cold H-50 Buffer (pH 7.0 20mM HEPES, 50mM KCl, 10mM NaCl, 1mM $MgSO_4$, 5mM $NaHCO_3$, 1mM NaH_2PO_4). Then the cells were resuspended in 100µl of cold H-50 buffer and the plasmids were transformed by electroporation using a gene pulser (75kv, 25µF) (BioRad, Hercules, CA). Transformants were selected with 10µg/ml G418 (geniticin; GIBCO BRL, Grand Island, NY). All the plasmids used in this study were introduced into *Dictyostelium* cells using electroporation.

5.1.2 Clone of *Dictyostelium* AP2α subunit and the generation of anti AP2α subunit polyclonal antibody

The *AP2A1* gene encoding the α subunit gene (~3.1kb) was identified from a *Dictyostelium* genome database (www.dictybase.org) using BLAST with the full amino acid sequence of mammalian α subunit gene. In the DH1 wild type cells we used in this study, there is only one copy of this *AP2A1* gene. The α subunit gene (*AP2A1*) was amplified from a cDNA library using the Polymerase Chain Reaction (PCR) with primers selected from the coding region. The 5' primer, 5'-CGGGGTACCATGAG-TATGAATGTTACAAATC-3' carried a KpnI site in the beginning while the 3' primer, 5-CGGGGTACCAGCAGCAGCAGCAGCAGCTTGTAAATGAGAGATTAATAA-ATT-3' carried a six alanine linker and also a KpnI cutting site. The resulting 3.1kb

piece was cloned into pTX-GFP expression vector using the KpnI site. The *AP2A1* gene was then subcloned from the pTX-GFP vector (Levi *et al.*, 2000) into the glutathione-S-transferase bacterial expression vector pGEX-2T (Smith and Johnson, 1988) using EcoRI and BamHI sites. GST-*AP2A1* was transformed into *E.coli* BL21 cells.

2L *E.coli* BL21 expressing the plasmid pGEX-2T-GST-*AP2A1* cells were induced at 37^{o}C with 0.5mM IPTG for 12 hours and the *E.coli* cells were collected and resuspended in 20ml lysis buffer (50mM Tris-HCL, pH7.5, 100mM NaCl, 5mM $MgCl_2$, 0.5% TritonX-100). Cell were lysed with a sonicator at 4 ^{o}C for 5 minutes. Then the supernatant of the bacterial cell lysates was collected and incubated in 2 ml lysis buffer with 75mg glutathione beads for 1 hour at 4 ^{o}C. The beads-cell lysate mix was washed with 10ml lysis buffer three times. The elution was collected with elution buffer (5mM reduced glutathione in lysis buffer) for three 1 ml fractions (O'Halloran and Anderson, 1992a). The purified protein was sent to Cocalico Biologicals (Reamstown, PA, USA) for immunization and generation of rabbit polyclonal antisera.

5.1.3 Generation of mutant cell lines using homologous recombination

I first generated a single AP2α null cell line in Ax2 wild type cells. Genomic sequences flanking the *Dictyostelium* α subunit gene, *AP2A1* were selected and amplified by Polymerase Chain Reaction (PCR). To amplify the ~1.08kb 5' flanking region of the *AP2A1* gene, I used primers 5'-CAAATTCAAAAACAACAA-GGAATACCCG-3' and 5'-GGGTGAAAGATTATCAA-ATGAA-TTGCAC-3'. To amplify the ~1.10kb 3' flanking region of the *AP2A1* gene, I used the primers 5'-TTATAACCACAACTCCCAAATCCTTTTTCAC-3' and 5'-CCCCAATACCACT TAAATAAATTGTTGC-3'. Each PCR product was initially cloned into the pCR2.1-

TOPO vector and then subcloned into the pSP72Bsr vector (Wang *et al.*, 2002), a derivative of pBluescriptII that encodes a 1.4kb blasticidin (Bsr) resistance gene. Then the resulting plasmid pSP72-Bsr-ΔAP2A1 was linearized with XhoI and EcoRV and introduced into wild type Ax2 cells by electroporation as described previously. Each transformation reaction was diluted into HL-5 medium with 5μg/ml Bsr and plated into six 96-well plates. Resulting clones were expanded and were assessed of the disruption of the gene by PCR and western blots.

Using the same pSP72-Bsr-ΔAP2A1 construct, I also generated an AP2α subunit null cell line in *Dictyostelium* DH1 wild type cells. In order to disrupt both *AP2A1* (gene encoding the α subunit of AP2) and *clmA* (the gene encoding AP180) or *epnA* (the gene encoding epsin) (Stavrou and O'Halloran, 2006; Brady *et al.*, 2008) in *Dictyostelium* cells, I first disrupted *AP2A1* gene in wild type DH1 cells. I subcloned the 5' (~1.08kb) and 3' (~1.10kb) flanking regions from the pSP72-Bsr-ΔAP2A1 construct into pSP72-pyr plasmid using the HindIII/XhoI and EcoRI sites respectively. The pSP72-pyr plasmid is a derivative of the pSP72BSR vector and has the blasticidin gene replaced by a ~1.5Kb *pyr* (pyrimidine biosynthetic) gene from the pRHI30 vector (Insall *et al.*, 1996). The resulting pSP72-pyr-ΔAP2A1, was linearized with XhoI and BglII, and introduced into wild type DH1 cells by electroporation. Each transformation reaction was diluted into FM minimal medium (Formedium LTD. Norwich England NR13, 4HY) and plated into six 96-well plates. Resulting clones were expanded and were assessed for the absence of AP2α subunit by western blot analysis.

To generate the AP2 α/AP180 double null cell line, I then further deleted the *clmA* gene in the AP2α subunit null cells. To do so, pSP72-Bsr-ΔAP180 (Stavrou and O'Halloran, 2006) was linearized with a BamHI and XhoI digestion and transformed in the AP2α subunit nulls cells via electroporation. Transformed cells were diluted in FM

minimal medium supplemented with 5μg/ml blasticidin (Bsr) and plated in 96-well plates. Resulting clones were screened for the absence of both AP180 and AP2α subunit by western blot analysis.

Using the same pSP72-Bsr-ΔAP180 construct, Irene Stavrou also generated the AP180 single null cell line in DH1 wild type cells by homologous recombination using electroporation. Transformed cells were selected in HL-5 media supplemented with 5μg/ml blasticidin and verified for the absence of the AP180 protein by western blot as described before.

For generating the AP2α/epsin double knock out cells, I deleted the *epnA* gene in the AP2α subunit null cells. To do so, pSP72-Bsr-EpsinKO (Brady *et al.*, 2008) was linearized using XhoI/HindIII and EcoRI digestion and transformed in the *AP2A1* nulls cells via electroporation. Transformed cells were diluted in FM minimal medium supplemented with 5μg/ml blasticidin (Bsr) and plated in 96-well plates. Resulting clones were screened for the absence of both epsin and AP2α subunit by western blot analysis. Using the same pSP72-Bsr-EpsinKO construct, Rebecca Brady also generated epsin single null cells in DH1 wild type cells by homologous recombination using electroporation. Transformed cells were selected in HL-5 media supplemented with 5μg/ml blasticidin and verified for the absence of the epsin protein by western blot as described before.

5.1.4 Clone of *Dictyostelium* AP2 μ2 subunit and the generation of the anti- μ2 antibody

The AP2 μ2 subunit gene (439bp) was identified from a *Dictyostelium* genome database (www.dictybase.org) using BLAST with the full amino acid sequence of mammalian μ2 subunit gene. The μ2 subunit gene was amplified from a cDNA library

using the Polymerase Chain Reaction (PCR) with primers selected from the coding region. The 5' primer is 5'-CGCGGATCCATGATTAGTGCA TTATTCTTAATG-3' and the 3' primer is 5'-TCCCCC GGGTTTTAAATACG ATTTTGATAGGTACCAG-3'. The result ~450 bp piece was subcloned into the TA cloning vector PCR2.1 (Invitrogen) first and then subcloned into the pTX-GFP vector (Levi *et al.*, 2000) using the BamHI site.

For generating the anti- μ2 polyclonal antibody, the μ2 gene was cloned from the pTX-GFP- μ2 into pMAL-C2X (NEW ENGLAND Biolabs, Ipswich, MA) using BamHI sites and was transformed into *E.coli* BL21 cells and the expressed protein was purified from bacterial lysates as previously described for generating the anti-α subunit antibody. The purified protein was sent to Cocalico Biologicals (Reamstown, PA, USA) for immunization and generation of rabbit polyclonal antisera.

5.1.5 Strains and cell culture

Dictyostelium discoideum wild type cells (Ax2 and DH1) and all mutant cells were grown on petri dishes in HL-5 nutrient media supplemented with 0.6% penicillin-streptomycin (GIBCO BRL, Gaithersburg, MD) at 20^0C. Clathrin heavy chain mutants were derived from Ax2 wild type cells and clathrin light chain mutants were derived from the wild type axenic strain NC4A2 (Niswonger and O'Halloran, 1997a; Wang *et al.*, 2003). AP2α subunit mutants and AP180 mutants used in the Chapter 2 were derived from Ax2 cells (Stavrou and O'Halloran, 2006). AP2α subunit mutants, AP180 mutants, epsin mutants, AP2 α/AP180 double mutants and AP2 α/epsin double mutants used in the Chapter 3 were derived from DH1 cells.

Cells expressing fluorescence plasmids were all maintained in HL-5 nutrient media supplemented with 0.6% penicillin-streptomycin and 10μg/ml G418 (geniticin;

GIBCO BRL, Grand Island, NY). All the plasmids in this study were introduced in *Dictyostelium* cells through electroporation as described before (Wang *et al.*, 2003).

pTX-GFP-AP180 was cloned by Irene Stavrou; pTX-epsin-RFP and pmmars-RFP-clathrin light chain constructs were cloned by Rebecca Brady; pTX-GFP-clathrin light chain was cloned by Jinshan Wang; pTX-GFP-Vamp7B (full length) and pTX-GFP-Vtil were cloned by Kevin Bersuker.

5.1.6 Western blot analysis

For western blots, cell lysates were prepared by resuspending cells into hot sample buffer. 1x106cells/lane were analyzed on 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (0.2 micron, BioRad, Hercules, CA). The membrane was probed with rabbit polyclonal antibody followed by a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase. Signal was detected using ECL kit (Pierce Biotechnology, Rockford, IL, USA). When more than one primary antibody was used, the membrane was washed three times with TBS (150mM NaCl, 50mM Tris, pH7.5) before probing with another antibody.

To compare the expression levels of clathrin and Vamp7B in wild type, AP2α null, AP180 null and AP2α/AP180 double null cell lines, ~1.6x107 cells from each cell line were first collected and washed once with PDF (2mM KCl, 1.1 mM K2HPO4, 1.32mM KH2PO4, 0.1mM CaCl2, 0.25 mM MgSO4, pH6.7). Then ~4x106 cells were taken from the total samples and lysed with 0.2% TritonX-100 at room temperature for 10 minutes. The protein concentration of all cell samples was measured using BioRad protein Assay (BioRad, Hercules, CA). The remaining unlysed (~1.2x107) cells of each cell line were spun down and resuspended in hot sample buffer to 20mg/ml according to the protein sample concentration. Using those samples the protein levels of clathrin

were detected by western blot using anti-clathrin heavy chain polyclonal antibody. Anti-aurora antibody was used (Li *et al.*, 2008) as the loading control for examining clathrin expression level, anti-myosin heavy chain antibody (Burns *et al.*, 1995) were used for examining expression level of pTX-GFP-Vamp7B.

5.1.7 Immunostaining and microscopy

Cells were allowed to attach to coverslips for 15 minutes. The attached cells were washed once with PDF buffer and flattened by overlaying a thin layer of 2% agar NA (Amersham Biosciences, Uppsala, Sweden). Then cells were fixed with 1% formaldehyde in 100% methanol at -20^{0}C for 5 minutes and blocked with 3% BSA in PBS at 37^{0}C for 15 minutes. For examining the localization of epsin or Hip1r, cells were fixed with a two step fixation protocol. Cells were fixed with 2% formaldehyde and 0.01% Triton-X 100 in PDF (2 mM KCL, 1.1 mM K_2HPO_4, 1.32 mM KH_2PO_4, 0.1 mM $CaCL_2$, 0.25 mM $MgSO_4$, pH 6.7) at room temperature for 15 minutes and then in 100% methanol at -20°C for 5 minutes.

Primary antibody was added to the fixed cells and cells were incubated at 37^{0}C for 45 minutes followed by four washes with phosphate-buffered saline (PBS). The cells were then incubated with secondary antibody with either a Texas Red tag or Pacific Blue tag (30µg/ml; Molecular Probes, Eugene, OR) at 37^{0}C for 45 minutes in dark. Following four gentle washes with PBS, the cells were rinsed briefly in distilled water and mounted on microscope slides with mounting media (MOWIOL, Calbiochem, EMD Biosciences, Inc. La Jolla, CA).

Anti-AP2α subunit antibody was prepared by pre-absorption as following: α subunit null cells were grown in suspension to a density of 2x10^{8}cells/ml. Cells were spun down at 1500 rpm for 5minutes and resuspended, in 2% formaldehyde in phosphate-

buffered saline (PBS) for 5 minutes at room temperature. The cells then were centrifuged at 2000 rpm for 5 minutes and resuspended in 1% formaldehyde in 100% methanol, incubated at -20^{0}C for 5 minutes. Next, the cells were centrifuged at 2000 rpm for 5 minutes and resuspended in 1.5 ml of 3% Bovine Serum Albumin (BSA) (Fisher Scientific, Fair Lawn, NJ) in PBS with 0.02% sodium azide. Anti-α subunit antiserum was added to prepared cells at a 1:500 dilution and incubated at 4^{0}C overnight. The supernatant of this antibody-cell mix was added to a new aliqout of prepared AP2α subunit null cells and incubated at 4^{0}C overnight. This was repeated at least three times. Clathrin light chain antibody was prepared in the same way as anti-α subunit antibody but using clathrin light chain null cells to pre-absorb. Affinity purified Hip1r antibody was a gift from Shannon Repass (Repass *et al.*, 2007). Monoclonal antibody p80 and polyclonal antibody Rh50 were gifts from Dr.Pierre Cosson.

Cells were visualized using differential interference contrast microscopy (DIC) and fluorescence microscopy on a NIKON Eclipse TE 200 microscope. Images were acquired on a Photometrics cooled CCD camera, processed using Metamorph 5.0 software (Universal Imaging Co. Downington, PA USA). For imaging contractile vacuole dynamics under DIC, cells were allowed to attach in microscopy chambers in nutrient media. Another set of cells were allowed to attach in chambers, first in nutrient media, and then in distilled water to provide a hypotonic environment. Images of living cells were captured every 6 seconds for about 10 minutes and compiled into quick time movies using Metamorph software played at 6 frames per second. Still images from timelapse microscopy were used to measure the diameter of fully expanded, round contractile vacuoles. The maximum diameter of contractile vacuoles was measured using the 100x calibrated distance tool in Metamorph software. Cells expressing and GFP-CLC were imaged in the same way except that low fluorescence media

(http:dictybase.org/techniques/media/lowfl o_medium.html) was used instead of nutrient HL-5 media and the movies were taken every 3 seconds instead of every 6 seconds.

Membrane-associated clathrin was quantified using a Leica scanning laser confocal microscope (TCS-SP2) and processed using Leica software. Images of cell membranes were taken under the Alexa 633 filter by focusing only on the top of the cells. Differential Interference Contrast (DIC) images were also used to make sure I was focusing on the top of the cell. Clathrin light chain null cells were used as a control for non-specific binding of the antibody and all images were scaled the same. To quantify the membrane association of clathrin, I collected images from 100 cells for each cell line. I used the Leica light software to circle the fluorescent area of each cell (outlining the plasma membrane) and then calculated the sum of fluorescence intensity of clathrin punctae on the cell surface. I then calculated the intensity over the area for each cell using Microsoft Excel and obtained an average for all the cells analyzed. The mean intensity/area value for each cell line was normalized against the background value of clathrin light chain cells. The value for wild type cells was used as the maximum (100%) intensity/area value. The difference in intensity/area between wildtype DH1, AP2α subunit null cells, AP180 null cells, AP2 α/AP180 double null cells and AP2 α/epsin double null cells were compared and standard errors between every cell within the same cell line were used as error bars.

5.1.8 Endocytosis assay

Fluid-phase endocytosis was measured by uptake FITC-Dextran (m.w 70kDa). 2mg/ml of FITC-Dextran was added to 3 x10^6 cells growing in suspension HL-5 medium. Samples were taken at 0, 15, 30, 60, 90 and 120 minutes and spun down at 1100 rpm at 4 ^{o}C. To stop the uptake, cells were washed twice and resuspended in HL-5 medium

containing 0.02% sodium azide and incubated on ice until all samples were collected. Then all samples were centrifuged at 1100rpm at 4 ^{o}C for 5 minutes and the cells were resuspended in cold Na_2HPO_4 buffer. After that, the cells were lysed with 20% Triton X-100 and fluorescence uptake was analyzed immediately by a BioRad VersaFluor fluorometer. Protein concentration of all cell samples were measured using BioRad protein Assay (BioRad, Hercules, CA) after lysis.

5.1.9 DAPI staining

For examining the growth in suspension, cells were diluted to $1x10^5$/ml and grown in HL-5 medium on a rotary shaker at 220 rpm at 18^0C for 72-84 hours. To examine growth on a substrate, cells were diluted to $1x10^5$/ml and grown on Petri dishes in HL-5 medium at 18^0C for 72-84 hours.

Cells were allowed to attach to coverslips for 15 minutes and washed once with PDF buffer and flattened using 2% agar NA. Then cells were fixed with 1% formaldehyde in methanol at -20^0C for 5 minutes as described before. Cells were then stained with 0.1% μg/ml DAPI (4,6-Diamidino-2-phenylindole) at room temperature for 10 minutes, followed by two washes with PBS. Then cells were mounted on microscope slides with mounting media and imaged using differential interference contrast microscopy and fluorescence microscopy on a NIKON Eclipse TE 200 microscope as described before.

5.1.10 Cell aggregation and development

To examine cell behavior during aggregation, $0.5x10^6$ cells were harvested and washed twice with 1xPDF. Then cells were resuspended in 1.5ml of PDF buffer and placed into 2 well glass chambers (Lab-Tek, Rochester, NY) at 18^0C for 15 to 21 hours

before imaging. For development on agar plates, 1-2x10^8 cells were harvested and washed once with starvation buffer (20mM MES, 0.2mM$CaCl_2$, 2mM$MgSO_4$) and resuspended to 2x10^7/ml in the same starvation buffer. 9 ml of the cell suspension was plated on 1% Noble agar (Difco Laboratories, Inc.) plates made with starvation buffer. The cells were allowed to attach on the plates for 30 minutes. After the liquid was aspirated from the plates, the plates were allowed to dry with the lid off for 40mins. Then the cells were incubated in dark at 18^0C for ~40 hours for observing the formation of fruiting bodies.

Spores were harvested from development plates by picking up individual fruiting body heads using sterile toothpicks and resuspending spores in PDF. Spores were then plated on glass coverslips and settled. Spores were imaged using NIKON Eclipse TE 200 microscope.

5.1.11 GST pull down assay

The *Dictyostelium* Vamp7B cytosolic domain is found within the N-terminus (amino acids 1-189 ;(Bogdanovic, *et al*., 2002). The first 1-186 amino acids of this cytosolic domain were amplified by the Polymerase Chain Reaction (PCR) using the Vamp7B full length sequence in the pTX-GFP-Vamp7B construct as a template. Primers were selected from the coding region of the gene: 5' primer, 5'-CGCGGATCCATGCC TATTATCTATTC-3' carried a BamHI site in the beginning, while the 3' primer, 5'-CGCGGATCCTCATTTCCACCACATTGCAC-3' carried a stop codon and also a BamHI cutting site. The resulting 558bp piece was cloned into into the glutathione-S-transferase bacterial expression vector pGEX-2T (Smith and Johnson, 1988) using EcoRI and BamHI sites. To express the GST-Vamp7B cytosolic domain, the plasmid was transformed into *E.coli* BL21 cells and the expressed protein was

purified from bacterial lysates using the same method as in purifying AP2α subunit previously described in 5.1.2.

For doing the GST pull-down assay, $5x10^6$ DH1 wild type cells expressing pTX-GFP-AP180 (Stavrou and O'Halloran, 2006) were collected and lysed in 1ml binding buffer (Vithalani *et al.*, 1998). 1ml of supernatant of the cell lysate was collected and incubated for 2 hours at 4^0C with 400µl Glutathione agarose beads saturated with purified Vamp7B cytosolic domain peptide. The beads were then collected, the flow-through from the beads were collected (the unbound fraction). The collected beads were washed 10 times with 1ml of the same binding buffer at 4^0C. Elution samples were collected by boiling the beads with 400µl hot sample buffer for 5 minutes. In western blots, 10 µl of the elution sample was loaded as the bound sample. 10µl of the flow-through solution was mixed with sample buffer and loaded as the unbound sample; and 10µl of the lysis sample was mixed with sample buffer and loaded. Beads saturated with GST protein and DH1 cells expressing only pTX-GFP were used as negative controls.

5.2 PLASMIDS, ANTIBODIES AND CELL LINES

Table 5.1: Plasmids used in this study

Plasmid	Description
pGEX-2T-AP2A1	Full length AP2α subunit cloned from cDNA into bacterial expression vector for protein purification and antibody production
pGEX-2T-Vamp7b(1-558bp)	Vamp7b cytosolic domain cloned into bacterial expression vector for protein purification and GST pull down assays
pMAL-C2X-AP2 μ2	Full length AP2 μ2 subunit cloned into bacterial expression vector for protein purification and antibody production
pTX-GFP-AP180	Full length AP180 cloned into pTX vector by Irene Stavrou. N-terminal GFP tag, G418 resistance (Stavrou and O'Halloran, 2006).
pTX-GFP-CLC	Full length clathrin light chain cloned into pTX vector by Jingshan Wang. N-terminal GFP tag, G418 resistance (Wang *et al.*, 2003).
pmmars-RFP-CLC	Full length clathrin light chain cloned into mRFPmars expression vector by Rebecca Brady. N-terminal RFP tag, Bsr resistance.

pTX-GFP- μ2	Full length AP2 μ2 subunit cloned into the pTX vector. N-terminal GFP tag, G418 resistance.
pTX-GFP-Vamp7B	Full length Vamp7B cloned into the pTX vector by Kevin Bersuker. N-terminal GFP tag, G418 resistance.
pTX-GFP-Vtil	Full length Vtil cloned into pTX vector by Kevin Bersuker. N-terminal GFP tag, G418 resistance.
pSP72-pyr-ΔAP2A1	1.08 kb *AP2A1* gene 5' UTR fragments and 1.10kb 3' UTR fragment flanking the pyrimidine biosynthetic (pyr) gene cassette to replace the *AP2A1* gene.
pSP72-Bsr-ΔAP180	1.3 kb *clmA* gene 5' UTR fragments and 1.6kb 3' UTR fragment flanking the blasticidin resistance gene cassette to replace the *clmA* gene (Stavrou and O'Halloran, 2006).
pSP72-Bsr-epsinKO	*epnA* gene 5' UTR fragments and 3' UTR fragment flanking the blasticidin resistance gene cassette to replace the *epnA* gene (Brady *et al.*, 2008).

Table 5.2 Cell lines and Antibodies used in this study

Cell lines	Description
Ax2	Wild type axenic strain, grows in HL-5 supplemented with 0.6% Pen Strep.
DH1	Derived from Ax3 wild type axenic strain, with the pyr5-6 gene depleted, uracil auxotroph, grow in HL-5 media but not minimum FM media.
AP2α null (6A5)	AP2α subunit null cells derived from Ax2 wild type strain, Bsr resistant.
AP2α null (3E1)	AP2α subunit null cells derived from DH1 wild type strain, uracil auxotroph, grow in HL-5 media and minimum FM media.
AP180 null (5H11)	AP180 null cells derived from Ax2 cells, Bsr resistant. Generated by Irene Stavrou (Stavrou and O'Halloran, 2006).
AP180 null (4B8)	AP180 null cells derived from DH1 cells, Bsr resistant, grow in HL-5 media but not FM media. Generated by Irene Stavrou.
Epsin null (4B1)	Epsin null cells derived from DH1 cells, Bsr resistant, grown in HL-5 media but not FM media. Generated by Rebecca Brady.
AP2 α/AP180 DKO(2H5)	AP2α subunit and AP180 double null cells derived from DH1 cells. Bsr resistant and can grow in both HL-5 and FM media.

AP2 α/epsin DKO(1A9)	AP2α subunit and epsin double null cells derived from DH1 cells. Bsr resistant and can grow in both HL-5 and FM media.
CHC null(5E2)	Clathrin heavy chain null cells derived from Ax2 wild type cells. Bsr resistant and grow in HL-5 media (Niswonger and O'Halloran, 1997a).

Antibodies	**Description**
Anti-AP2α subunit	Rabbit polyclonal antibody (UT371). Use 1:2000 for western blot. Used at 1:500 for immunostaining after pre-absorped with AP2α null cells. This antibody is NOT stable for immunostaining, store at 4 ^{0}C up to 1 month after pre-absorption.
Anti-AP2 mu2 subunit	Rabbit polyclonal antibody (UT451). Use 1:2000 for western blot. I have not tested it for immunostaining.
Anti-AP180	Rabbit polyclonal antibody made by Irene Stavrou. Use the UT 325 test bleed, number 4 (UT325-4) 1:2000 for western blot.
Anti-Hip1r	Affinity purified polyclonal rabbit anti-Hip1r antibody, made by Shannon Repass and used

	at 1μg/ml for immunostaining (Repass *et al.*, 2007).
Anti-CLC	Rabbit polyclonal anti clathrin light chain antibody. Used 1:1000 for western blot. Used at 1:500 for immunostaining after pre-absorption with clathrin light chain null cells.
Anti-Rh50	Purified rabbit polyclonal antibody Rh50 is a gift from Dr. Pierre Cosson. Used at 1:1000 for immunostaining
Anti-p80	Monoclonal antibody p80 is a gift from Dr. Pierre Cosson. Used at 1:500 for immunostaining
Anti-GFP	Rabbit polyclonal antibody was generated by Hui Li. Used at 1:2000 for western blots
Anti-Aurora	Rabbit polyclonal antibody was generated by Hui Li (Li *et al.*, 2008). Used at 1:2000 for western blots
Anti-MHC	Rabbit polyclonal antibody (Burns *et al.*, 1995). Used at 1:2000 for western blots.

Appendices

The DPF AP2 binding motif is not necessary for AP180 to bind AP2 in Dictyostelium

AP180 orthologs contain a DPW/DPF motif which has been shown to bind to the both α and β2 subunits of AP2 (Iannolo *et al.*, 1997; Owen *et al.*, 1999; Owen *et al.*, 2000). This direct binding between AP2 and AP180 accounts for their cooperative clathrin assembly activity. *Dictyostelium* AP180 has only one DPF motif. To test whether this DPF motif is essential to the binding between AP2 and AP180, two amino acids of this motif were mutated (from DPF to APA) and a plasmid expressing mutated AP180 was transformed into AP180- cells (Stavrou and O'Halloran, unpublished data).

I first examined whether this mutated AP180 had a physical interaction with AP2 *in vivo* using both immunoprecipitation and immunostaining microscopy. Surprisingly, AP180- cells with this mutated motif still co-immunoprecipitated with AP2 (FigD1 A) and colocalized with AP2 on both plasma membrane and on contractile vacuoles. Furthermore, these cells with mutated AP180 still had wild type size contractile vacuoles (FigD1 B,C,D). All these results highly suggest that in *Dictyostelium* cells, the DPF motif is not required either for binding AP2 or for controlling the size of contractile vacuoles.

Thus there must be an unidentified AP2 binding motif in *Dictyostelium* AP180. Earlier studies have suggested that motifs in clathrin adaptor proteins, such as DxF/W, FxDxF and Wxx (F/W) x (D/E) could also bind to AP2 (Brett *et al.*, 2002; Mishra *et al.*, 2004; Praefcke *et al.*, 2004). I searched the *Dictyostelium* AP180 sequence and found three DxF (x, any amino acids) sequences which may be able to serve as AP2 binding site substitute in the DPF mutant cells.

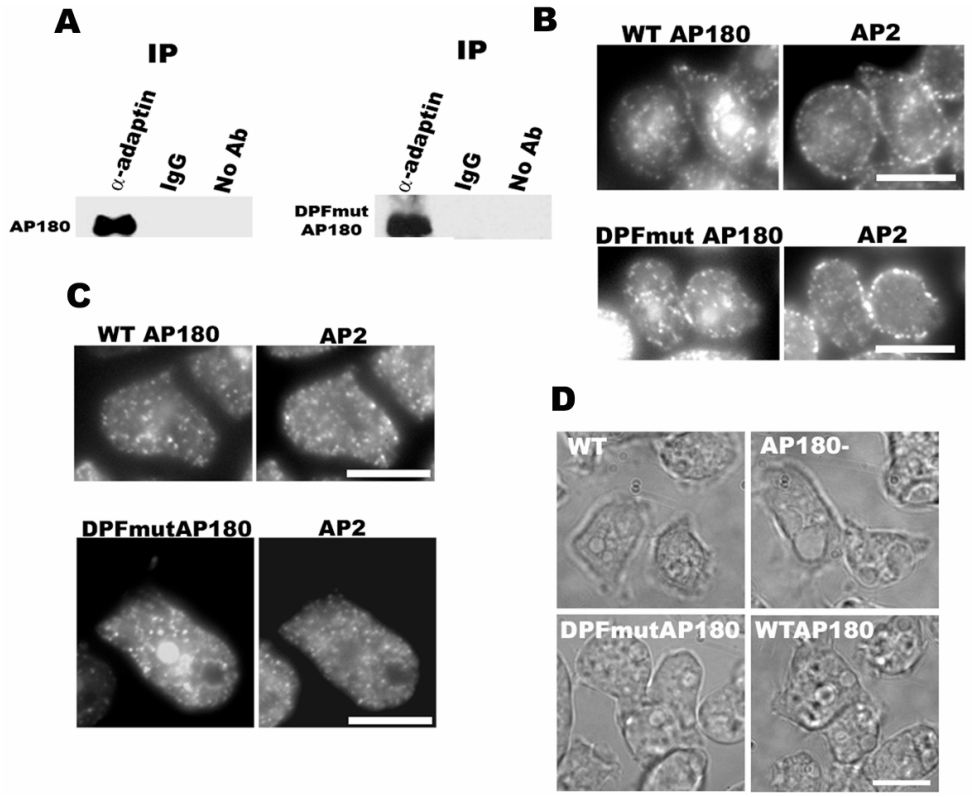


Figure 1D. AP2 DPF binding motif on *Dictyostelium* AP180 is not required for AP180 function or the association with AP2. (A). Immunoprecipitation experiments shows that AP180 with a mutated DPF motif (DPF->APA) can still associate with AP2 *in vivo*. Wild type cells expressing either wild type AP180(AP180) or AP180 with a mutated DPF motif (DPF mutant AP180) were lysed. Whole cell lysates were then incubated with polyclonal anti-AP2α antibody. (B). AP180 with a mutated DPF motif can still colocalize with AP2 at the plasma membrane. Cell expressing either GFP tagged wild type AP180 (AP180) or GFP-tagged AP180 with a mutated DPF motif (DPF mutant AP180) were fixed and immunostained with anti-AP2α antibody. (C). AP180 with a mutated DPF motif can still colocalize with AP2 at the plasma membrane. Cell expressing either GFP-tagged wild type AP180 (AP180) or GFP-tagged AP180 with a mutated DPF motif (DPF mutant AP180) were fixed and immunostained with anti-AP2α antibody. (D). The enlarged contractile vacuoles in the AP180 null cells were rescued by wild type AP180 as well as AP180 with a mutated DPF.

TIRF—Is AP2 or AP180 involved in regulating actin dynamics during clathrin mediated endocytosis?

Dynamic actin is required in the late stage of clathrin coated pits formation and pits internalization. Rebecca Brady has showed that in *Dictyostelium* cells, epsin and Hip1r are required for actin polymerization at the clathrin internalization sites. In the absence of either of epsin or Hip1r cells, actin punctae are diffuse and mobile on the plasma membrane. In addition, the association between actin punctae and clathrin punctae at the plasma membrane are also reduced and clathrin punctae take longer time to internalize in epsin or Hip1r null cells (Brady and O'Halloran, unpublished data).

To investigate whether other clathrin adaptor proteins AP2 and AP180 are also required for the actin dynamic during clathrin mediated endocytosis, I examined wild type cells and AP2α/AP180 double mutant cells co-expressing an actin marker LimE Δ coil tagged with GFP and RFP-clathrin light chain using TIRF microscopy. In wild type cells, plasma membrane associated clathrin coated vesicles internalized with clathrin puncta quickly disappearing from the cell surface. During this period of time, there was very little lateral movement of the clathrin punctae. Frequently, this disappearance of clathrin puncta was accompanied by the appearance of LimE Δ coil GFP labeled actin puncta. In contrast to the wild type cells, in AP2 α/AP180 double null cells, actin puncta were laterally mobile and clathrin puncta spent more time on the plasma membrane before they got internalized (movie data not shown). This phenotype resembles epsin and Hip1r null cells suggesting AP2 or/and AP180 could also be involved in regulating actin dynamic, directly or indirectly.

However, I have not been able to examine AP2α subunit or AP180 single null cells to see whether AP2 and/or AP180 was responsible for this phenotype.

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Vita

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