Dynamics of clathrin-mediated endocytosis and its requirement for organelle biogenesis in Dictyostelium

Laura Macro, Jyoti K. Jaiswal* and Sanford M. Simon†

Laboratory of Cellular Biophysics, The Rockefeller University, New York, NY 10065, USA

*Present address: Center for Genetic Medicine Research, Children's National Medical Center, Washington DC, DC 20010, USA

†Author for correspondence (simon@rockefeller.edu)

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Summary

The protein clathrin mediates one of the major pathways of endocytosis from the extracellular milieu and plasma membrane. In single-cell eukaryotes, such as Saccharomyces cerevisiae, the gene encoding clathrin is not an essential gene, raising the question of whether clathrin conveys specific advantages for multicellularity. Furthermore, in contrast to mammalian cells, endocytosis in S. cerevisiae is not dependent on either clathrin or adaptor protein 2 (AP2), an endocytic adaptor molecule. In this study, we investigated the requirement for components of clathrin-mediated endocytosis (CME) in another unicellular organism, the amoeba Dictyostelium. We identified a heterotetrameric AP2 complex in Dictyostelium that is similar to that which is found in higher eukaryotes. By simultaneously imaging fluorescently tagged clathrin and AP2, we found that, similar to higher eukaryotes, these proteins colocalized to membrane puncta that move into the cell together. In addition, the contractile vacuole marker protein, dajumin-green fluorescent protein (GFP), is trafficked via the cell membrane and internalized by CME in a clathrin-dependent, AP2-independent mechanism. This pathway is distinct from other endocytic mechanisms in Dictyostelium. Our finding that CME is required for the internalization of contractile vacuole proteins from the cell membrane explains the contractile vacuole biogenesis defect in Dictyostelium cells lacking clathrin. Our results also suggest that the machinery for CME and its role in organelle maintenance appeared early during eukaryotic evolution. We hypothesize that dependence of endocytosis on specific components of the CME pathway evolved later, as demonstrated by internalization independent of AP2 function.

Key words: Clathrin, AP2, Dictyostelium

Introduction

Clathrin-mediated endocytosis (CME) is one of the major mechanisms for internalization of nutrients, signaling molecules and transmembrane receptors from the extracellular milieu and plasma membrane (McMahon and Boucrot, 2011). Although clathrin is required in metazoans, the gene encoding clathrin is not an essential gene in single-cell eukaryotes, such as Saccharomyces cerevisiae and Dictyostelium (Payne and Schekman, 1985; Lemmon and Jones, 1987; O’Halloran and Anderson, 1992; Ruscetti et al., 1994; Niswonger and O’Halloran, 1997a; Niswonger and O’Halloran, 1997b; Wang et al., 2003). Given that clathrin functions at numerous membrane transport steps, it has been difficult to resolve whether CME from the plasma membrane is important for any of these processes.

Evidence for CME in Dictyostelium have revealed its importance for macropinoscytosis, cytokinesis, development and osmoregulation (O’Halloran and Anderson, 1992; Ruscetti et al., 1994; Niswonger and O’Halloran, 1997a; Niswonger and O’Halloran, 1997b; Wang et al., 2003). Given that clathrin puncta have been observed to colocalize with AP2, epsin, Hip1r and AP180 (Stavrou and O’Halloran, 2006; Repass et al., 2007; Brady et al., 2008; Wen et al., 2009; Sosa et al., 2012). In the case of AP2, immunofluorescence studies using an antibody to the α or β1/2 subunits showed that they localize to clathrin puncta (Wen et al., 2009; Sosa et al., 2012). In addition, the β1/2 subunit has been shown to interact with the μ2 subunit (Sosa et al., 2012); however, the existence of a full heterotetrameric AP2 complex similar to that found in mammalian cells has not been established.

In Dictyostelium, clathrin puncta have been observed to disappear at the plasma membrane coincident with a burst of actin (Brady et al., 2010). Although disappearance of clathrin from the membrane is a consequence of endocytosis,
disappearance as the sole criteria for endocytosis is insufficient because it is also observed when the clathrin coat disassembles during uncoating and as a result of photobleaching (Ehrlich et al., 2004; Merrifield et al., 2005; Mattheyses et al., 2011). Thus, a robust measure for CME requires demonstration of cargo internalization together with the disappearance of clathrin from the cell membrane, as seen in mammalian systems (Ehrlich et al., 2004; Merrifield et al., 2005; Rappoport et al., 2005; Mattheyses et al., 2011). Similar evidence of internalization of specific plasma membrane cargo via CME has been lacking in Dictyostelium. Furthermore, owing to the use of low temporal resolution and fixed-cell imaging for monitoring CME in Dictyostelium, there is limited dynamic information about the endocytosis of clathrin-coated vesicles.

In Dictyostelium, loss of expression of many different endocytic proteins results in a defect in osmoregulation, which is controlled by a specialized organelle called the contractile vacuole (Gerisch et al., 2002). Osmoregulation phenotypes of varying severity are observed in both clathrin light chain (clc–) (Wang et al., 2003) and heavy chain (chc–) knockouts (O’Halloran and Anderson, 1992), as well as in knockouts of AP180 (Stavrou and O’Halloran, 2006), the γ, β1/2 or µ2 subunits of AP2 (Wen et al., 2009; Sosa et al., 2012), and the µ1 subunit of AP1 (Lefkir et al., 2003). Although the function of clathrin and its adaptors at the contractile vacuole is unclear, mislocalization of contractile vacuole components in AP1 and AP2 knockouts has suggested roles for these complexes in contractile vacuole biogenesis (Lefkir et al., 2003; Sosa et al., 2012).

To study CME vesicles in live cells, we identified a cargo protein and determined the members of the AP2 complex in Dictyostelium. Using biochemical and optical tools, we found that cargo, clathrin and AP2 in Dictyostelium have dynamics characteristic of endocytosis. The AP2 complex colocalizes with clathrin in plasma membrane puncta that internalize from the cell surface into the cytosol. Our results provide direct evidence for the existence of a clathrin-mediated pathway for endocytosis in Dictyostelium, detailed characterization of the kinetics of clathrin and AP2-containing vesicles, and establish a role for this pathway in the biogenesis of the contractile vacuole.

**Results**

**Cloning and characterization of the Dictyostelium AP2 complex**

Genes corresponding to the subunits of AP1, AP2, AP3 and AP4 in Dictyostelium were inferred from homology to their mammalian counterparts (supplementary material Table S1). Experimental evidence has confirmed these annotations for the γ, β1/2 and µ1 subunits of AP1 (Lefkir et al., 2003), for the 6, 3β and 3µ subunits of AP3 (Bennett et al., 2008) and for the β1/2 and µ2 subunits of AP2 (Sosa et al., 2012). To investigate the AP2 complex in Dictyostelium, we cloned the Ap2A1 gene and labeled it with green fluorescent protein (GFP) at the N terminus (GFP-Ap2A1). Expression of GFP-Ap2A1 in wild-type cells (AX2) resulted in the expression of a single 140-kDa protein, in agreement with the predicted size for GFP-Ap2A1 (Fig. 1A). Expression of this protein showed no detectable effect on doubling time or time taken by cells to undergo development (growth curves; development on agar; data not shown). To identify proteins that interact with GFP-Ap2A1 and form the AP2 complex, cells expressing GFP-Ap2A1 were lysed and incubated with an anti-GFP antibody. The co-immunoprecipitated proteins were separated by SDS-PAGE and the proteins identified by mass spectrometry. The proteins included the predicted α, β, µ and σ subunits of the AP2 complex (Fig. 1B, supplementary material Table S2). We identified the protein product of the gene Ap1B1 as the β subunit, independently confirming the recent report that AP1 and AP2 use the same β subunit in Dictyostelium (Sosa et al., 2012). We also identified the protein products of the apm2 gene as the µ2 subunit and of the Ap2S1 gene as the σ2 subunit; other AP complex subunits were not identified. We also identified the Dictyostelium homolog of Eps15, a clathrin adaptor known to bind to the AP2 α subunit in mammalian cells (van Delft et al., 1997). These results demonstrate the existence of a functional AP2 complex in Dictyostelium that is homologous to the AP2 complex found in mammals. Additionally, GFP-Ap2A1 was incorporated into this AP2 complex and, at the level of expression used here, did not localize to any other protein complexes in the cell.

**Intracellular localization and dynamics of clathrin and AP2 in Dictyostelium**

Using GFP-Ap2A1 as a reporter for the AP2 complex, we investigated its localization within the cell. Confocal microscopy showed that GFP-Ap2A1 localized to distinct puncta that were distributed around the cell periphery (Fig. 2A), in agreement with a previous study that used antibodies raised against Ap2A1 (Wen et al., 2009). Imaging of cells by total internal reflection fluorescence microscopy (TIR-FM), which excites molecules within ~100 nm of the coverslip, enabled us to establish that AP2 puncta are at, or close to, the plasma membrane (Fig. 2B). TIR-FM time-lapse imaging of GFP-Ap2A1 revealed the dynamic nature of AP2 puncta: puncta appeared at the
membrane, remained there for various lengths of time and then disappeared from the TIR-FM field (Fig. 2D). Occasionally, AP2 puncta could be seen to localize in rings or circles (supplementary material Fig. S1A), closely resembling AP2 puncta localization on bladders of the contractile vacuole (Wen et al., 2009) and, thus, were excluded from further analysis.

Fig. 2. Intracellular localization and dynamics of clathrin and AP2. (A) Confocal sections showing the intracellular localization of GFP-Ap2A1 in wild-type (AX2), Ap2A1−, apm2−, clc− and chcA− cells. (B) TIR-FM images of GFP-Ap2A1 puncta in AX2, Ap2A1−, apm2−, clc− and chcA− cells. (C) TIR-FM images of mRFP-clc puncta in AX2, Ap2A1−, apm2−, chcA− and GFP-clc puncta in clc− cells. (D) Examples of the dynamics of individual GFP-Ap2A1 puncta imaged by TIR-FM in AX2, chcA− and apm2− cells. Normalized fluorescence intensity is plotted as a function of time for each punctum. (E) Examples of the dynamics of individual mRFP-clc puncta imaged by TIR-FM in AX2, Ap2A1− and apm2− cells. Normalized fluorescence intensity is plotted as a function of time for each punctum. (F–H) Cell surface duration of (F) GFP-clc puncta in clc− cells; (G) GFP-Ap2A1 puncta in AX2 cells; and (H) GFP-Ap2A1 in chcA− cells. (I) Percentage of GFP-Ap2A1 puncta present at the end of the time-lapse with durations >350 seconds. Scale bars: 1 μm (A–C); 0.5 μm (D,E). See also supplementary material Fig. S1 and Table S3.
When AP2 puncta were imaged continuously for 7.5 seconds, three different puncta behaviors were observed: 78±8% remained static, 16±8% disappeared and 7±5% moved laterally (mean ± s.d.; n=363). When AP2 puncta were followed over a longer time period (time-lapse for 10 minutes), all the puncta observed to appear on the surface disappeared within 6 minutes, with an average duration on the surface of 56.3±4.2 seconds (mean ± s.e.m.; n=111) (Fig. 2G). Some puncta were lost owing to the movement of cells during the course of imaging and could not be included in the kinetic analysis. In an attempt to remove any potential bias in our manual tracking, we tested the use of automated tracking to follow every punctum in the cell. This method gave large errors and a significantly shorter duration because it did not exclude puncta that disappeared owing to cell movement. Thus, because of their highly motile nature, automated tracking is not a suitable approach to analyze the kinetics of endocytic puncta in *Dictyostelium*.

To compare the behavior of AP2 with clathrin, we imaged clathrin light chain-knockout cells (*clc–*) expressing GFP-tagged clathrin light chain (GFP-clc) (Wen et al., 2009) and performed the same analysis. In TIR-FM, GFP-clc localized to distinct membrane puncta as well as to contractile vacuoles, as previously observed (Fig. 2C; supplementary material Fig. S1A) (Brady et al., 2010; Wen et al., 2009). Continuous imaging of clathrin puncta for a 7.5-second period revealed their dynamic behavior, with 55±18% remaining static, 26±14% disappearing and 19±5% moving laterally (mean ± s.d.; n=300). When clathrin puncta were imaged over a longer time period (time-lapse for 10 minutes), all puncta appeared on the surface disappeared within 5 minutes, with an average duration on the surface of 55.6±6.0 seconds (mean ± s.e.m.; n=103) (Fig. 2F). This is similar to the value of 39 seconds previously reported (Brady et al., 2010).

Comparison of clathrin and AP2 puncta showed that they had similar distributions of duration times at the cell surface (Fig. 2F,G); however, more clathrin puncta had shorter durations at the cell surface (21% of clathrin puncta have a lifetime <10 seconds versus 0% of AP2 puncta). We believe that this population corresponds to clathrin-coated intracellular vesicles that move close to the plasma membrane during imaging, thus being detected using TIR-FM (Keyel et al., 2004). Using a two-sample Kolmogorov–Smirnov (KS) test, we saw that, if the puncta with durations <10 seconds were removed from the clathrin population, the distribution of duration times was no longer significantly different between AP2 and clathrin (KS test, P=0.066) (supplementary material Table S3).

The localization of clathrin in dynamic membrane puncta at the cell membrane was consistent with results from mammalian cells and showed that, in *Dictyostelium*, clathrin-coated pits form on the plasma membrane. Our results also showed that, in *Dictyostelium*, the localization and dynamics of AP2 are similar to those in mammalian cells and, therefore, AP2 puncta correspond to clathrin-coated pits.

**Clathrin heavy chain is required for the dynamic behavior of AP2 puncta**

The formation and maturation of a clathrin-coated pit involves the recruitment of a complex array of proteins. It has been difficult to study the requirements for individual components of the clathrin-coated vesicle cycle in mammalian cells because of redundancy and incomplete knockdowns. We took advantage of the availability of clathrin and AP2 knockouts in *Dictyostelium* to investigate whether they are required for the observed cell surface dynamics of puncta.

We expressed GFP-Ap2A1 in cells with either disrupted clathrin light chain (*clc–*) (Wang et al., 2003) or disrupted clathrin heavy chain (*chcA–*) (Ruscetti et al., 1994). TIR-FM imaging of GFP-Ap2A1 in *clc–* cells showed that AP2 was able to localize to membrane puncta that behaved with similar dynamics to wild-type cells (Fig. 2A,B; supplementary material Fig. S1C,E).

In *chcA–* cells, although GFP-Ap2A1 was able to form membrane puncta with a normal appearance, there was an increased number of diffuse puncta compared with wild-type cells (Fig. 2B). In addition, there was cell-to-cell variability, with some cells showing no puncta. We analyzed AP2 puncta by fitting a single-peak two-dimensional (2D) Gaussian curve to individual puncta, the width of the Gaussian where it falls to 1/e (mean ± s.d.; n=391) compared with 2.9±0.8 pixels in wild-type cells (n=461); this difference was statistically significant (t-test, P<0.001). Confocal imaging of GFP-Ap2A1 in *chcA–* cells showed that, although GFP-Ap2A1 could bind to the membrane, the puncta often stuck together and localized to one side of the cell (Fig. 2A). Furthermore, time-lapse TIR-FM imaging showed high variability in the behavior of individual AP2 puncta; several puncta were very short lived (durations <10 seconds), some had dynamics indistinguishable from the wild type, whereas others were long lived (present at the beginning of imaging and did not disappear for over a 12-minute period) (Fig. 2D). Analysis of the time for which the dynamic GFP-Ap2A1 puncta were detectable on the surface showed an increased proportion of puncta with durations of <10 seconds, causing a significantly shorter average surface duration of 15.2±2.0 seconds (mean ± s.e.m.; n=127) compared with wild-type cells (52.3±4.2 seconds) (Fig. 2H); correspondingly, the distribution of durations was significantly different between wild-type and *chcA–* cells (KS test, P=3.80×10^{-10}). To analyze the long-lived puncta, we quantified the time of arrival of puncta present at the end of the time-lapse to determine how many had a duration greater than 350 seconds. For wild-type cells, we did not observe any puncta with a duration >350 seconds; however, in *chcA–* cells, we found that 43.3% of the GFP-Ap2A1 puncta had a duration >350 seconds. This number was also elevated in *clc–* cells (26.3%) (Fig. 2I). This shows that, although clathrin is not required for the plasma membrane localization of AP2, its loss results in the appearance of two abnormal AP2 puncta populations, one that is very short lived and one that is long lived. This suggests that clathrin has roles in both stabilizing AP2 binding at the membrane and the progression of AP2 puncta to internalization.

**AP2 µ2 is not required for AP2 α puncta formation**

We next investigated the effects of disrupting the α (*Ap2A1–*) (Wen et al., 2009) or µ2 (*apm2–*) (Mercanti et al., 2006b) subunits on the localization and dynamics of AP2 or clathrin in cells (Fig. 2B,C). In both the α and µ2 knockouts, clathrin puncta at the plasma membrane were similar to wild-type cells (Fig. 2C,E; supplementary material Fig. S1B,G; Table S3). TIR-FM imaging of GFP-Ap2A1 in *Ap2A1–* cells showed some
puncta with dynamics in the range of wild-type cells; however, the distribution of durations was significantly different (KS test, $P=0.0094$) because of the appearance of a population with shorter durations (<20 seconds) (supplementary material Fig. S1C,E; Table S3). This could be because of an incomplete rescue of the Ap2A1 phenotype by the GFP-Ap2A1 protein. Interestingly, in m2 knockouts, GFP-Ap2A1 also localized to distinct membrane puncta, some of which behaved with dynamics in the range of wild-type cells. A population with shorter durations also appeared, making the distributions significantly different (KS test, $P=2.23\times10^{-5}$) (supplementary material Fig. S1C,E; Table S3). This could be because of an incomplete rescue of the Ap2A1– phenotype by the GFP-Ap2A1 protein.

Interestingly, in m2 knockouts, GFP-Ap2A1 also localized to distinct membrane puncta, some of which behaved with dynamics in the range of wild-type cells. A population with shorter durations also appeared, making the distributions significantly different (KS test, $P=2.23\times10^{-5}$) (Fig. 2B,D; supplementary material Fig. S1C,E; Table S3). Confocal imaging showed that, although GFP-Ap2A1 puncta could be seen on the plasma membrane in apm2– cells, there was a higher cytoplasmic level of GFP-Ap2A1 (Fig. 2A). Western blot of cell lysates showed that loss of m2 does not destabilize the $\alpha$ subunit (Fig. 1A), consistent with the finding that the $\beta1/2$ subunit is also stable in apm2– cells (Sosa et al., 2012). To investigate the composition of the GFP-Ap2A1 puncta, we incubated lysates from apm2– cells expressing GFP-Ap2A1 with an anti-GFP antibody and separated and identified the co-immunoprecipitated proteins using SDS-PAGE and mass spectrometry (Fig. 1C). We found that, in the absence of the m2 subunit, GFP-Ap2A1 was still able to interact with the $\beta1/2$ (Ap1B1) and $\alpha2$ (Ap2S1) subunits of the AP2 complex. Although we did not find any of the other AP m subunits, we did identify the $\gamma$ subunit of AP1 (Ap1G1). These results indicate that, in the absence of the $\mu$2 subunit in Dictyostelium, a partial AP2 complex can form that contains the $\gamma$ subunit.

Clathrin and AP2 puncta colocalize and disappear together

To test the hypothesis that clathrin and AP2 puncta corresponded to clathrin-coated pits, we investigated their behavior in the same cell. Both mRFP-clc and GFP-Ap2A1 colocalized in puncta at the plasma membrane in live cells, in agreement with observations in fixed cells (Fig. 3A) (Wen et al., 2009). Colocalization analysis demonstrated that 76±18% of AP2 puncta were positive for clathrin and 57±19% of clathrin puncta were positive for AP2 (mean ± s.d.; $n=33$ cells) (Fig. 3C). Colocalization of less than 100% is consistent with observations of clathrin and AP2 in mammalian cells (Rappoport et al., 2003).

Two color TIR-FM time-lapse imaging of puncta showed that GFP-Ap2A1 and mRFP-clc displayed similar dynamics at the plasma membrane (Fig. 3B,D). Individual puncta were analyzed by calculating the time of disappearance from the TIR field for both GFP-Ap2A1 and mRFP-clc ($n=132$). This analysis showed that, for 58% of puncta, AP2 disappeared within 4 seconds of clathrin disappearance; thus, we conclude that AP2 and clathrin leave the TIR field at similar times and behave with similar dynamics at the plasma membrane.

Clathrin and AP2 disappearance corresponds to endocytosis

To further investigate the nature of AP2 and clathrin disappearance, we monitored these puncta by alternating between widefield (EPI) and TIR. Given that the TIR field decays with a space constant of ~100 nm but the EPI field has a focal depth of ~500 nm, puncta that are internalized should continue to be detected with EPI illumination after disappearing from the TIR field (Merrifield et al., 2002; Mattheyes et al., 2011).

Cells expressing GFP-Ap2A1 and clc-mRFP were imaged by alternating EPI and TIR illumination and the fluorescence intensity of individual puncta was quantified for each channel and used to group puncta based on their behavior (Fig. 4). Puncta

**Fig. 3.** Simultaneous imaging of the dynamics of clathrin and AP2. (A) Two-color TIR-FM images of GFP-Ap2A1 and mRFP-clc puncta. Scale bar: 2 μm. (B) Example of dynamics of an individual punctum imaged by TIR-FM. Scale bar: 0.5 μm. (C) Quantification of colocalization between GFP-Ap2A1 and mRFP-clc puncta imaged by TIR-FM. The percentage overlap is plotted for GFP–Ap2A1 versus mRFP-clc (gray bar) and for mRFP-clc versus GFP-Ap2A1 (white bar). Data are represented as mean ± s.d. ($n=33$ cells). (D) Normalized fluorescence intensity for the punctum shown in B is plotted as a function of time for GFP-Ap2A1 (green, open circles) and mRFP-clc (red, filled circles). (E) Time between mRFP-clc and GFP-Ap2A1 disappearance from the TIR-FM field ($n=132$ puncta).
were only analyzed if, by TIR, they could be seen in both the GFP (AP2) and RFP (clathrin) channels, and the fluorescence signal accumulated in a punctum followed by a rapid disappearance from TIR. For some puncta, the clathrin EPI signal could not be detected above the noise; in these cases, only the AP2 EPI signal was used for analysis. A vesicle that formed in TIR, pinched off the membrane and moved into the cell, consistent with endocytosis, was observed for 32±17% of puncta (mean ± s.d.; n=93) (Fig. 4A). For these puncta, the fluorescence appeared in EPI simultaneously with, or soon after, the TIR signal, consistent with the de novo formation of a clathrin-coated pit at the plasma membrane, and inconsistent with an internal vesicle moving towards the cell surface. When these puncta subsequently disappeared from the TIR field, they continued to be observed in EPI illumination, indicating movement away from the plasma membrane into the cell.

For 17±13% of puncta, the EPI signal appeared and disappeared simultaneously with the signal in TIR (Fig. 4B). These events could correspond to endocytic vesicles that either lose their clathrin and AP2 coincident with the vesicle leaving the plasma membrane, or to vesicles internalizing faster than the imaging speed. Alternatively, the loss of signal could be because of an unproductive clathrin-coated pit where clathrin and AP2 disassemble at the cell membrane. Both of these behaviors have been reported in mammalian cells (Mattheyses et al., 2011). A small percentage (12±6%) of puncta had an EPI signal that appeared before the TIR signal and, thus, appeared to be internal vesicles that entered the TIR field (Fig. 4C) and not clathrin-coated de novo. In 39±18% of puncta, the EPI signal was not bright enough to distinguish from the background fluorescence in the cytoplasm, hence their fate could not be classified. We expect that the behavior of these puncta will be equally distributed between the behaviors observed above and so conclude that most (>50%) AP2 and clathrin puncta that disappeared from the TIR field showed behavior that corresponded to productive endocytic events.

**Dajumin-GFP is a plasma membrane cargo for clathrin-mediated endocytosis**

Given that clathrin and AP2 have a role in osmoregulation in Dictyostelium (Wen et al., 2009; O’Halloran and Anderson, 1992), we examined whether CME is involved in retrieval of the contractile vacuole proteins that might have entered the cell membrane. Although contractile vacuoles are thought to fuse with the plasma membrane by a kiss-and-run mechanism that does not involve any membrane mixing (Heuser, 2006; Gabriel et al., 1999), it is possible that there is a low level of leakage into the plasma membrane upon pore formation (Wen et al., 2009; Gabriel et al., 1999; Sosa et al., 2012).

We coexpressed the contractile vacuole specific marker protein dajumin-GFP (Gabriel et al., 1999) with mRFP-clc and imaged cells with TIR-FM (Fig. 5A). Dajumin-GFP labeling of the contractile vacuole could be seen as bright bladders and tubules close to the basal membrane, as described previously (Gabriel et al., 1999). Closer inspection of our images revealed that dajumin-GFP also localized to dim puncta that colocalized with mRFP-clc puncta (Fig. 5A). Colocalization analysis showed that 40±14% of mRFP-clc overlapped with dajumin-GFP and 22±12% of dajumin-GFP overlapped with mRFP-clc (mean ± s.d.; n=25 cells) (Fig. 5B). Colocalization was validated by performing a pixel shift analysis showing that the peak
colocalization overlap was in the nonshifted image (dx=0) (Fig. 5C). Colocalization quantification was confounded by the relatively dim fluorescence of dajumin-GFP puncta; they appeared and disappeared and were not present at each time point. As a result, there was considerable cell-to-cell variability in the percentage overlap.

In cells expressing Ap2A1 tagged with mRFPmars (mRFP-Ap2A1) and dajumin-GFP (Fig. 5D), we found, by TIR-FM, dajumin-GFP puncta that colocalized with mRFP-Ap2A1. Colocalization analysis showed 16±6% of mRFP-Ap2A1 overlapped with dajumin-GFP and 10±8% of dajumin-GFP overlapped with mRFP-Ap2A1 (mean ± s.d.; n=45 cells).
Again, there was cell-to-cell variability and colocalization was validated using pixel shift analysis (Fig. 5F). The colocalization of this transmembrane protein with AP2 and clathrin suggested that it was internalized in clathrin-coated vesicles.

To investigate the dynamics of dajumin-GFP puncta, we used time-lapse imaging of cells expressing dajumin-GFP and mRFP-clc (Fig. 5G). Puncta were tracked over time and the fluorescence intensity was quantified (Fig. 5H). Dajumin-GFP accumulated with clathrin followed by a rapid disappearance from TIR. The time of initiation of disappearance from the TIR field was calculated for both dajumin-GFP and mRFP-clc for each punctum (n=47) (Fig. 5I). This analysis showed that 47% of dajumin-GFP puncta disappeared together with clathrin (<4 seconds apart). Given that we showed that clathrin puncta that disappeared from TIR moved into the cell (Fig. 4), we conclude that dajumin-GFP is internalized from the plasma membrane of Dictyostelium via CME.

Clathrin heavy chain is essential for internalization of plasma membrane dajumin-GFP

Internalization of dajumin-GFP from the plasma membrane was confirmed using a biotin internalization assay (Barth et al., 1994) (Fig. 6A). Plasma membrane proteins of wild-type cells...
expressing dajumin-GFP were reversibly biotinylated with a membrane-impermeable biotin. Immunoprecipitation of dajumin-GFP from cell lysates, followed by SDS-PAGE and western blot analysis with streptavidin-HRP, showed that dajumin-GFP is biotinylated and, thus, present on the plasma membrane. Western blot analysis of dajumin-GFP showed that the full-length protein and two cleavage products (45 kDa and 40 kDa) were present in cells (Gabriel et al., 1999). Tagging of dajumin on its extracellular domain confirmed that these are products of cleavage and indicated the protein is cleaved at, or close to, the transmembrane domain (data not shown). Only the full-length protein and the 45-kDa fragment were biotinylated; the 40-kDa fragment was either not on the plasma membrane or did not have extracellular amino groups accessible to the biotinylation reagent.

We next followed the fate of cell surface dajumin-GFP by allowing the cells to internalize proteins for 15 minutes by incubation at 22°C. Residual cell surface biotin was removed by incubation with a membrane-impermeable reducing agent. As a control, some cells were kept on ice and, thus, did not undergo any cell surface internalization. The results show that following the internalization step at 22°C, the amount of biotinylated dajumin-GFP increased, demonstrating endocytosis from the cell surface.

We assessed the requirement of clathrin and AP2 for internalization by expressing dajumin-GFP in Ap2A1–, apm2–, clec– and chcA– cells. In Ap2A1–, apm2– and clec– cells, the amount of biotinylated dajumin-GFP increased following the internalization step, showing that plasma membrane dajumin-GFP was internalized similar to wild-type cells. By contrast, in chcA– cells, dajumin-GFP was biotinylated and, therefore, was on the plasma membrane, but no internalization was ever observed (Fig. 6A,B).

We confirmed these results by imaging the localization of dajumin-GFP in the various cell lines (Fig. 6C). In wild-type, Ap2A1–, apm2– and clec– cells, deconvolution of z-stacks showed the predominant localization of dajumin-GFP to be in contractile vacuoles, in agreement with recently published results (Sosa et al., 2012). The additional plasma membrane localization of dajumin-GFP that can be seen in individual focal planes has, however, not previously been described. In chcA– cells, no contractile vacuoles were observed (O’Halloran and Anderson, 1992) and dajumin-GFP was predominantly localized to the plasma membrane (Fig. 6B), again in agreement with (Sosa et al., 2012). The absence of contractile vacuoles in chcA– cells was confirmed by staining with the lipophilic dye FM4–64 (Heuser et al., 1993) and with an antibody to the vacuolar proton pump (VatM) (Fok et al., 1993) (supplementary material Fig. S2). Some internal dajumin-GFP positive structures could be seen; these did not colocalize with a fluid-phase endocytic marker or a lysosomal marker (10 kDa TxR-dextran; Lysotracker DND Blue; data not shown) and might represent either a rudimentary contractile vacuole that has not fused with the plasma membrane or the dajumin-GFP transiting through the Golgi compartment.

Together, our results show the existence of a clathrin-mediated endocytic pathway via which plasma membrane dajumin-GFP is internalized in a clathrin-dependent, AP2-independent mechanism. This suggests that internalization from the plasma membrane by clathrin-coated vesicles is important for the biogenesis and maintenance of the contractile vacuole in Dictyostelium.

**Discussion**

The formation of clathrin-coated vesicles is characterized by the successive binding of various proteins. The adaptor complex AP2 acts as a central player in this process by binding to clathrin, many other accessory proteins and cargo (McMahon and Boucrot, 2011). We showed that a heterotetrameric AP2 complex exists in Dictyostelium that localizes to internalizing clathrin-coated pits on the plasma membrane. Given that AP1 and AP2 use the same β subunit in Dictyostelium (Sosa et al., 2012), and the AP1 complex has been shown to localize to the Golgi (Lefkir et al., 2003), membrane specificity of the AP complexes is not conferred by the β subunit. This is consistent with studies of mammalian AP complexes, which showed that β subunits primarily have a role in clathrin binding rather than membrane specificity (Page and Robinson, 1995; Shih et al., 1995).

In Dictyostelium, clathrin and AP2 showed similar dynamics at the membrane and, when imaged simultaneously, they colocalized in puncta and disappeared from the TIR-FM field together. This signature is consistent with the behavior of AP2 and clathrin puncta in mammalian cells (Rappoport et al., 2006). For both AP2 and clathrin puncta, there was a variety of durations on the plasma membrane, again a property consistent with mammalian clathrin-coated pits (Mattheyses et al., 2011). We took advantage of the spatial selectivity of TIR-FM compared with wide-field illumination to image the internalization of clathrin-coated pits as they moved from the plasma membrane into the cell. Taken together, our results demonstrated that AP2 and clathrin accumulate at clathrin-coated pits that disappear from the TIR-FM field, corresponding to productive endocytic events in Dictyostelium.

Deletions in clathrin and AP2 genes in Dictyostelium were used to study the requirements for these proteins in clathrin-coated vesicle formation. Our results showed that AP2 can form membrane puncta in the absence of clathrin heavy chain but that some of these puncta cannot progress to internalization, similar to observations in mammalian cells (Henne et al., 2010). These results suggest that the progressive nature and precise timing of forming a clathrin-coated vesicle have been conserved throughout evolution. Furthermore, we also observed a population of AP2 puncta that has a much shorter surface duration, indicating that clathrin also has a role in stabilizing AP2 at the membrane.

In the absence of the AP2 μ2 subunit, GFP-Ap2A1 was stable and able to localize to plasma membrane puncta of partial AP2 complexes. This suggests that the AP2 complex can assemble and target to the plasma membrane without the μ2 subunit, consistent with findings for AP1 in Dictyostelium (Lefkir et al., 2003). It is possible that the AP complexes are more dynamic than previously thought, with subunits functioning independently or exchanging freely. Interestingly, we also detected the γ subunit of AP1 in our co-immunoprecipitation. Given that AP1 and AP2 use the same β subunit, it is possible that we detected the γ subunit via this interaction. The appearance of a short-lived population of AP2 puncta in the μ2 and γ knockouts suggested that the individual subunits have a role in stabilizing the binding of the AP2 complex to the membrane.

Given that clathrin mutants are not lethal in unicellular organisms, such as Dictyostelium and yeast (O’Halloran and Anderson, 1992; Payne and Schekman, 1985; Lemmon and Jones, 1987), it has been suggested that CME acts as a nonspecialized endocytic portal in these lower eukaryotes (McMahon and Boucrot, 2011). For the first time in Dictyostelium, we have shown that a specific transmembrane cargo, dajumin-GFP, is internalized via CME. Just as clathrin is
required for internalization of many cargos in mammalian cells, internalization of dajumin-GFP is fully dependent on clathrin. Interestingly, its uptake is not dependent on AP2 suggesting that, in Dictyostelium, AP2 is not essential to form clathrin-coated vesicles, a property normally associated with CME in mammalian cells (Motley et al., 2003; Henne et al., 2010; Boucot et al., 2010). Endocytosis independent of AP2 function is also seen in yeast (Huang et al., 1999; Yeung et al., 1999); thus, dependence on AP2 might have arisen in metazoans. However, AP2 function might not have been fully removed in apm2–knockouts owing to the presence of a partial AP2 complex that is still able to localize properly. Selection of dajumin-GFP as cargo might depend on the adaptor AP180, which has already been shown to have a role in retrieving Vamp7B from contractile vacuoles, limiting their homotypic fusion (Wen et al., 2009).

A role for clathrin and AP1 in contractile vacuole biogenesis is well documented (O’Halloran and Anderson, 1992; Wang et al., 2003; Lefkir et al., 2003; Sosa et al., 2012). In AP1 μl–knockouts, the contractile vacuole is completely absent, and the contractive vacuole proteins Rh50 and dajumin-GFP are mislocalized to punctate structures inside the cell (Lefkir et al., 2003; Sosa et al., 2012), indicating that at least some contractive vacuole components are trafficked in a clathrin-coated vesicle-dependent mechanism. Dajumin-GFP localization to the plasma membrane in cheA– and β1/2-knockout cells was previously reported (Sosa et al., 2012); this study suggests that AP2 only functions in sorting of dajumin-GFP in the context of an AP1 knockout. In agreement, we found that there was no observed defect in dajumin-GFP trafficking in AP2 knockouts alone; however, we showed that dajumin-GFP traffics through the plasma membrane even in wild-type cells, suggesting that this pathway operates normally, and not only in the context of mis-sorting by a AP1 knockout.

Our data showed a role for CME in the biogenesis and/or maintenance of the contractile vacuole by functioning in retrieval of proteins from the cell membrane. This is in agreement with results for Rh50 showing that a fusion protein with the cytoplasmic tail of Rh50 traffics via the plasma membrane en route to the contractile vacuole (Mercanti et al., 2006a). It also suggests the plasma membrane is a potential source of membrane for the contractive vacuole. It is possible that CME arose as a mechanism of protein sorting and has evolved to serve other functions in higher eukaryotes. Further identification of cargoes internalized by CME in Dictyostelium will help in addressing whether it performs only a sorting function in this organism.

In addition to its biosynthetic route, dajumin-GFP might enter the plasma membrane upon emptying of the contractile vacuole. Membrane mixing and entry of dajumin-GFP to the plasma membrane is not observed upon kiss-and-run fusion pore formation (Heuser, 2006; Gabriel et al., 1999). However, CME might function as a back-up mechanism in case of membrane mixing. It is unclear whether CME has any role in the regulation of contractive vacuole emptying and pore formation itself.

Our results offer the first direct demonstration that clathrin is utilized for endocytosis of membrane cargo in Dictyostelium in a process distinct from macropinocytosis and phagocytosis. This pathway is distinguished by the generation of endocytic vesicles that contain AP2, clathrin and dajumin. Furthermore, our analysis of this pathway at a high spatial and temporal resolution shows the high degree of similarity in the kinetics of internalization of individual clathrin-coated vesicles between Dictyostelium and mammalian cells. These similarities support the conclusion that the formation of clathrin-coated vesicles is homologous between Dictyostelium and mammals and that Dictyostelium is a useful model to study CME in vivo.

Materials and Methods

Plasmid construction

To generate mRFPmars-Ap2A1 (mRFP-Ap2A1), the coding region of Ap2A1 was amplified from a cDNA library and inserted into the vector 339-3 (Fischer et al., 2004) (provided by Dr A. Müller-Taubenberger) to generate GFP-Ap2A1, mRFPmars was replaced with GFP. Either GFP-Ap2A1 or mRFP-Ap2A1 was inserted into pSM304, pDM326 and/or pDM358 (Veltman et al., 2009) to allow selection with other resistance markers.

To generate mRFPmars-clc (mRFP-clc), the clc sequence from pTX-GFP-clc (Wang et al., 2006) (provided by Dr T. O’Halloran) was inserted into 339-3, mRFP-clc was then inserted into pDM304 for expression. To generate clc-mRFPmars (clc-mRFP), first mRFPmars was inserted into pDM304 or pDM326 to generate pDM304-mRFP(C) and pDM326-mRFP(C). The cclc sequence was then inserted into pDM304-mRFP(C) and pDM326-mRFP(C).

The plasmid encoding dajumin-GFP was provided by Dr A. Müller-Taubenberger (Gabriel et al., 1999). Dajumin was inserted into pDM326-mGFP(C) to generate dajumin-mGFP.
There was a plateau in the decline of the fluorescence intensity. Kymographs were created using a maximum intensity projection of the four pixels either side of the center of the spot for every time point. Classification of puncta into various categories was done manually by assessing both the fluorescence intensity measurements and kymographs for each punctum.

**Biotinylation assay**

The assay was performed as described in Barth et al. (Barth et al., 1994). 5×10^5 cells were used per time point. After the cell surface reduction step, cells were lysed in 50–100 μl of lysis buffer and total protein concentration measured. Equal amounts of total protein were used for co-immunoprecipitation as described above, except 10–20 μl of GFP-Trap-A beads were used and incubation was performed for 2 hours at 4°C. Samples were eluted from the bead in 30–60 μl of non-reducing sample buffer. For detecting GFP, western blots were performed as described above; 5–25 μl of the sample was run on a 4–20% Tris-glycine gel followed by transfer to nitrocellulose membranes. For detection of biotin, 20 μl of the sample was run on a 4–20% Tris-glycine gel followed by transfer to nitrocellulose membranes. Membranes were blocked for 1 hour with 1% BSA Fraction V in wash buffer (150 mM NaCl, 50 mM Na2B4O7, pH 10.0) and then probed for 1 hour with Pierce High Sensitivity Streptavidin-HRP (1:8,000) in 4% BSA Fraction V in wash buffer containing 0.02% Triton-X100. Membranes were washed three times with wash buffer containing 0.1% Triton-X100, followed by three washes with wash buffer containing 0.1% Triton-X100. HRP was visualized using ECL Plus.

**Immunofluorescence**

Cells were incubated in imaging media on 25 nm NO. 1.5 glass coverslips and fixed as described in (Fok et al., 1993) but without the agar overlay. Cells were then immunostained (Clarke et al., 1987) with the N4 antibody (Fok et al., 1993) (1:50) followed by a goat anti-mouse AF488 secondary antibody (1:400). Z-slices were taken with a widefield microscope with a 100×1.35 NA objective and deconvolved with the software Huygens using calculated PSFs. Samples were excited with a xenon lamp and a 470/40 excitation filter, a GFP dichroic mirror was used with a 520/40 emission filter.

**FM4-64 staining**

Cells were incubated in imaging media on MatTek dishes and FM4-64 dye was added at 1 μg/ml for 20 min. Cells were imaged with the DeltaVision microscope described above.

**Acknowledgements**

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**Supplementary material available online at**


**References**


**Fig. S1. Dynamics of clathrin and AP2 (related to Fig. 2).** (A) TIR-FM images of mRFP-clc and GFP-Ap2A1 showing examples of puncta localized in rings most likely around contractile vacuole bladders. Scale bar = 1 μm. (B-C) Box charts showing the durations of XFP-clc puncta (B) or GFP-Ap2A1 puncta (C) in the different cell lines. Crosses = maximum and minimum values; solid square = mean; error bars = s.d.; box = 25, 50 and 75 percentile. (D-E) Histograms of the duration on the surface for XFP-clc puncta (D) or GFP-Ap2A1 puncta (E) in the different cell lines. The mean duration ± s.e.m and the number of puncta measured are given.
Fig. S2. Localization of contractile vacuole markers in AP2 and clathrin knockouts (related to Fig. 6). (A) Widefield microscopy images of FM4-64 labeling in AX2, Ap2A1-, apm2-, clc- and chcA- cells and corresponding DIC images. (B) Maximum intensity projection of VatM immunostaining in AX2, Ap2A1-, apm2-, clc- and chcA- cells. Scale bars = 1 μm.
<table>
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<tr>
<th>Name</th>
<th>Gene</th>
<th>Dictybase Gene ID</th>
<th>Percent identity with human (name and accession number)</th>
<th>Divergence</th>
<th>Experimental evidence</th>
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<tr>
<td>AP-1 γ subunit</td>
<td>ap1g1</td>
<td>DDB_G0281957</td>
<td>44.4 (AP-1 gamma-1 isoform b, NP_001119)</td>
<td>84.2</td>
<td>(Lefkir et al., 2003)</td>
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<td>AP-1 β subunit</td>
<td>ap1b1</td>
<td>DDB_G0279141</td>
<td>52.2 (AP-1 beta-1 isoform b, NP_663782)</td>
<td>68.4</td>
<td>(Sosa et al., 2012)</td>
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<td>67.4 (AP-1 mu-1 isoform 2, NP_115882)</td>
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<td>(Lefkir et al., 2003)</td>
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<td>AP-1 σ 1 subunit</td>
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<td>52.6</td>
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<td>AP-3 δ subunit</td>
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<td>AP-3 β subunit</td>
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<td>113.5</td>
<td>(Bennett et al., 2008)</td>
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<td>DDB_G0274003</td>
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The genes annotated as belonging to AP complexes in *Dictostelium* (www.dictybase.org) are shown. Homologous human proteins were identified by searching GenBank using BLASTP (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and the *Dictostelium* AP complex protein sequences. The top hit was used to calculate percent identity and phylogenetic divergence using the Clustal V method and MegAlign 9 software.
Table S2. Summary of LC-MS/MS Results (related to Fig. 1).

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<th>Protein</th>
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<th>apm2- + GFP-Ap2A1</th>
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<tr>
<td></td>
<td></td>
<td>Number of unique peptides</td>
<td>Percent coverage</td>
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<td>ap1b1 (β subunit)</td>
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<td>35 (103)</td>
<td>41%</td>
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<td>apm2 (µ subunit)</td>
<td>G0277139</td>
<td>13 (71)</td>
<td>31%</td>
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<td>ap2s1 (σ subunit)</td>
<td>G0289721</td>
<td>7 (36)</td>
<td>44%</td>
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<tr>
<td>ap1g1 (γ subunit)</td>
<td>G0281957</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td>eps15</td>
<td>G0287325</td>
<td>34 (118)</td>
<td>41%</td>
</tr>
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Summary of LC-MS/MS data for proteins identified from the gels shown in Fig. 1B,C.
Table S3. Kolmogorov-Smirnov (KS) test P values (related to Fig. 2 and Fig. S1).

KS test was performed to ask whether the distribution of the durations of cell surface puncta measured in different cell lines are drawn from the same distribution.

<table>
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<th>Data Set 2</th>
<th>KS P value</th>
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<td>GFP-clc (clc-)</td>
<td>GFP-Ap2A1 (AX2)</td>
<td>0.04594</td>
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<td>GFP-clc minus 10s (clc-)</td>
<td>GFP-Ap2A1 (AX2)</td>
<td>0.06564</td>
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<tr>
<td>mRFP-clc (clc-)</td>
<td>GFP-Ap2A1 (AX2)</td>
<td>2.15915 x 10^{-8}</td>
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<td>mRFP-clc minus 10s (clc-)</td>
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### Table S4. List of strains and expression plasmids used.

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<td>339-3 (Bsn)</td>
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<tr>
<td>apm2-/A15]:GFP:Ap2A1</td>
<td>pDM304 (G418)</td>
</tr>
<tr>
<td>clc-/A15]:GFP:Ap2A1</td>
<td>pDM304 (G418)</td>
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<tr>
<td>chcA-/A15]:GFP:Ap2A1</td>
<td>pDM304 (G418)</td>
</tr>
<tr>
<td>AX2/[A15]:mRFP:clc</td>
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<tr>
<td>Ap2A1-/A15]: mRFP:clc</td>
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<td>AX2/[A15]:dajumin:GFP [A15]:mRFP:clc</td>
<td>pDEXRH (G418)</td>
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<tr>
<td>chcA-/A15]: dajumin:GFP</td>
<td>pDEXRH (G418)</td>
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</tbody>
</table>

Strain shows the genetic background and the promoter used to drive expression. Plasmids used for expression are listed along with the antibiotic resistance used for selection. Bsn = blasticidin, G418 = geneticin, Hgr = hygromycin.