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The AP-1 Clathrin-adaptor Is Required for Lysosomal Enzymes Sorting and Biogenesis of the Contractile Vacuole Complex in *Dictyostelium* Cells

Yaya Lefkir,* Benoît de Chassey,* Annick Dubois,* Aleksandra Bogdanovic,† Rebecca J. Brady,‡ Olivier Destaing,§ Franz Bruckert,† Theresa J. O'Halloran,‡ Pierre Cosson,¶ and François Letourneur*#

*Institut de Biologie et Chimie des Protéines, UMR5086, CNRS/Université Lyon I, IFR 128 BioSciences Lyon-Gerland, 7, Passage du Vercors, 69367 Lyon cedex 07, France; †Laboratoire de Biochimie et Biophysique des Systémes Intégrés, 38054 Grenoble Cedex 9, France; ‡241 Patterson Laboratories, Section of Molecular Cell and Developmental Biology, The University of Texas at Austin, Austin, Texas 78712; §Laboratoire de Biologie Moléculaire et Cellulaire/UMR 5665 Ecole Normale Supérieure de Lyon, 69364 Lyon Cedex 07, France; and ¶Université de Genève, Centre Médical Universitaire, Département de Morphologie, CH-1211 Genève 4, Switzerland

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Adaptor protein complexes (AP) are major components of the cytoplasmic coat found on clathrin-coated vesicles. Here, we report the molecular and functional characterization of *Dictyostelium* clathrin-associated AP-1 complex, which in mammalian cells, participates mainly in budding of clathrin-coated vesicles from the *trans*-Golgi network (TGN). The γ-adaptin AP-1 subunit was cloned and shown to belong to a Golgi-localized 300-kDa protein complex. Time-lapse analysis of cells expressing γ-adaptin tagged with the green-fluorescent protein demonstrates the dynamics of AP-1–coated structures leaving the Golgi apparatus and rarely moving toward the TGN. Targeted disruption of the AP-1 medium chain results in viable cells displaying a severe growth defect and a delayed developmental cycle compared with parental cells. Lysosomal enzymes are constitutively secreted as precursors, suggesting that protein transport between the TGN and lysosomes is defective. Although endocytic protein markers are correctly localized to endosomal compartments, morphological and ultrastructural studies reveal the absence of large endosomal vacuoles and an increased number of small vacuoles. In addition, the function of the contractile vacuole complex (CV), an osmoregulatory organelle is impaired and some CV components are not correctly targeted.

INTRODUCTION

Transport of proteins between compartments of the endocytic and secretory pathways occurs mainly via vesicles coated with specific cytosolic proteins (Mellman, 1996; Rothman and Wieland, 1996). One of the best characterized coat protein is clathrin, which, in association with adaptor complexes (APs), participates in multiple transport steps (Hirst and Robinson, 1998; Smith and Pearse, 1999).

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Corresponding author. E-mail address: f.letourneur@ibcp.fr.

Four different AP complexes have been identified (AP-1 to AP-4; Boehm and Bonifacino, 2001). All APs share a similar composition. They are comprised of two large subunits (80–130 kDa), one medium size subunit (50 kDa), and one small subunit (20 kDa). For instance, AP-1 contains β1- and γ-adaptin large chains, a μ1A or μ1B medium chain, and a σ1A or σ1B small chain (Scales *et al.*, 2000). Cargo sorting relies on β-adaptin and μ chains. These two chains recognize tyrosine- and leucine-based sorting signals found in the cytoplasmic domains of cargo proteins (Ohno *et al.*, 1995; Rapoport *et al.*, 1998), thereby concentrating them into budding vesicles.

Each individual AP complex functions at a distinct intracellular site (Kirchhausen, 1999). Hence, AP-1 is mainly re-

cruited to membranes of the *trans*-Golgi network (TGN) and participates in transport from the TGN to endocytic compartments. Recently, the function of AP-1 in membrane traffic has been carefully reassessed. Gene targeted disruption of μ1A or γ-adaptin in mice and of μ1 in *Saccharomyces cerevisiae* suggested that AP-1 is required for retrograde transport from endosomes to the TGN of mannose 6-phosphate receptors (MPR) in mammalian cells as well as chitin synthase III and syntaxin Tlg1p in yeast cells (Zizioli *et al.*, 1999; Meyer *et al.*, 2000; Valdivia *et al.*, 2002). However, studies on the dynamics of AP-1 followed in living cells expressing yellow-fluorescent-protein–tagged μ1 revealed that AP-1 is mainly associated with transport structures moving from the TGN toward the cell periphery, whereas AP-1–associated retrograde transport appears to rarely occur (Huang *et al.*, 2001). In addition, AP-1 has been implicated both in the transport of the transferrin receptor from apical to basolateral membranes in epithelial cells (Futter *et al.*, 1998) and the recycling of the low-density lipoprotein receptor and the transferrin receptor to the basolateral membrane (Gan *et al.*, 2002). AP-1 was also reported to mediate transport from the TGN to the basolateral membrane of many membrane proteins (Folsch *et al.*, 1999, 2001). Thus AP-1 could function in more than one location; however, how a single vesicular coat could play multiple roles is not understood yet.

The amoeba *Dictyostelium discoideum* is a genetically tractable eukaryotic cell that is commonly used as cellular model to study membrane trafficking in the endocytic pathways. Being a professional phagocyte, *Dictyostelium* binds to and internalizes large size particles (>1 μm) by phagocytosis (Maniak, 1999; Neuhaus and Soldati, 1999; Cardelli, 2001; Maniak, 2001; Rupper and Cardelli, 2001). Laboratory strains can also grow in axenic liquid culture medium. Fluid-phase nutrients are then mainly internalized by macropinocytosis, concentrated in endosomes, and degraded in lysosomes. Undigested material can eventually be returned to the cell surface via postlysosomal vacuoles (Neuhaus and Soldati, 1999). The biosynthesis of lysosomal enzymes in *Dictyostelium* is similar to that in mammalian cells (Cardelli, 1993). Newly synthesized lysosomal hydrolases are first synthesized as membrane-bound, N-glycosylated precursor proteins in the ER and then transported to the Golgi. However, in contrast to mammalian cells where lysosomal enzymes are targeted to lysosomes through the recognition of mannose 6-phosphate (M6P) sugars by MPRs, the sorting machinery recognizing M6P sugars is poorly characterized in *Dictyostelium*, and in particular MPRs have not been identified yet (Cardelli, 1993). The fact that the clathrin heavy chain is required for proper sorting of lysosomal enzymes (Ruscetti *et al.*, 1994) suggests that clathrin-coated vesicles play a crucial role in this process in *Dictyostelium*.

As a first step toward a better understanding of AP-1–dependent transport mechanisms, we decided to investigate AP-1 function in the model organism *Dictyostelium*. Here we report for the first time the molecular and functional characterization of the AP-1 complex in this organism. Together our data provide new evidence for the sorting function of AP-1 in the endocytic pathway and uncover the role of AP-1 in CV biogenesis.

MATERIALS AND METHODS

Cell Culture, Development, and Secretion Assay

D. discoideum strain DH1–10 (Cornillon *et al.*, 2000) was cultured at 22°C in HL5 medium. To measure growth on bacterial lawns, cells were mixed with 400 μl of overnight liquid culture of *Klebsiella aerogenes* and plated on SM nutrient agar plates (Kay, 1987). For developmental analysis, cells were plated on 0.45-μm membrane filters (Whatman, Maidstone, UK) laid on Na/K phosphate buffer and incubated at 24°C in humid chambers (Sussman, 1987). To assess constitutive secretion in HL5 culture medium, cells were resuspended in HL5 at 5×10^6 cells/ml and incubated at 22°C. After 10 h, culture medium was TCA precipitated and pellets were analyzed by Western blotting.

Electron Microscopy

For conventional electron microscopy the cells were processed as described (Orci *et al.*, 1973). Sections were photographed in a Philips CM-10 transmission electron microscope (Philips, Eindhoven, The Netherlands) at calibrated magnifications.

Antibodies and Immunofluorescence Microscopy

Polyclonal antibodies to *Dictyostelium* μ1 and γ-adaptin were raised in rabbits using KLH-coupled peptides (^{320}VPPDADTPKFRC^{331}; Covalab, Lyon, France) and GST-γ (592–896) recombinant protein, respectively. Rabbit polyclonal antibodies to *Dictyostelium* cathepsin D (Journet *et al.*, 1999) and clathrin heavy chain (CHC) were gifts from Dr. J. Garin (CEA, Grenoble, France) and Dr. T. O'Halloran (University of Texas, Austin, TX), respectively. Mouse monoclonal antibodies (mAb) against α-mannosidase (2H9; Mierendorf *et al.*, 1983), comitin (190-68-1; Weiner *et al.*, 1993), and a Golgi unknown antigen (1/39; Graf *et al.*, 1999), were kind gifts from Dr. H. Freeze (University of California, La Jolla, CA), Dr. A. Noegel (University of Köln, Germany), and Dr. R. Gräf (Muenchen, Germany). Monoclonal antibodies (mAbs) against coronin (176-2-5), vacuolin B (221-1-1), and the A subunit of the vacuolar H^+-ATPase (221-35-2) have been characterized previously (Zhu and Clarke, 1992; Jenne *et al.*, 1998; Neuhaus *et al.*, 1998; de Hostos, 1999). The rat antiphagosome mAb M12A9 was reported to cross-react with *Dictyostelium* γ-adaptin (Morrissette *et al.*, 1999). The mAb H161, specific for a p80 cell surface marker, has been described (Ravanel *et al.*, 2001). Rh50 was stained with a rabbit polyclonal antibody (Benghezal *et al.*, 2001). To allow simultaneous labeling with an anti-p80 antibody and another mouse mAb, the H161 antibody was directly coupled to Alexa Fluor 488 (Molecular Probes, Leiden, Netherlands) according to manufacturer's instructions, and cells were processed for immunofluorescence as described (Ravanel *et al.*, 2001). For indirect immunofluorescence analysis, unless indicated, cells were grown on glass coverslips for 3 days, fixed with −20°C methanol for 10 min, incubated with the indicated antibodies for 1 h, and then stained with corresponding fluorescent secondary antibodies. Cells were visualized with a Zeiss confocal microscope (LSM510) (LePecq, France). For fluorescence microscopy in living cells, just prior observation cells were compressed under a thin layer of agarose (Yumura *et al.*, 1984). For excitation of all GFP constructs, the 488 band of an argon-ion laser was used together with a 515–565 filter for emission. For epifluorescence microscopy cells were observed using a Zeiss Axioplan 2 microscope.

Immunoprecipitation, Binding Assay, and Western Blotting

For immunoprecipitations, 10^7 cells were lysed in lysis buffer (PBS, 10 mM EDTA, 1% Triton X-100, protease inhibitors) and cleared by centrifugation for 15 min at 13,000 rpm in a microfuge. About 1 mg of protein extract was incubated overnight at 4°C with mAb M12A9 and Gammabind Sepharose beads (Amersham Pharmacia, Orsay,

France). The beads were then washed three times in lysis buffer and twice in 50 mM Tris-HCl, pH 7.5, and bound proteins analyzed by immunoblots. SDS polyacrylamide electrophoresis and immunoblotting were performed as previously described (Cornillon *et al.*, 2000). Bands were visualized using an ECF kit (Amersham Pharmacia) and a STORM Imager (Molecular Dynamics, Sunnyvale, CA). Binding assays between GST-γ fusion proteins and cytosol were carried out as described (Doray and Kornfeld, 2001).

Gel Filtration, Sucrose Gradient Fractionation

To prepare cytosol, *Dictyostelium* cells were washed twice in breaking buffer (1 mM EDTA, 150 mM NaCl, 20 mM MES-Na buffer, pH 6.5, 10 mM iodoacetamide, protease inhibitor cocktail), suspended at 4×10^8 cells/ml in the same buffer and then broken by six strokes in a ball-bearing cell cracker (Balch and Rothman, 1985). Unbroken cells were removed by low speed centrifugation ($1000 \times g$, 5 min, 4°C). The supernatant was then centrifuged at $100,000 \times g$ for 1 h in a SW50Ti rotor. Cytosol was collected and then loaded onto a HiPrep 16/60 Sephacryl S-300 HR gel filtration column (Amersham Pharmacia) equilibrated in breaking buffer. One-milliliter fractions were collected, and protein contents were TCA precipitated before SDS-PAGE and immunoblotting analysis. Membrane preparations and sucrose gradient fractionation were carried out as reported (Bogdanovic *et al.*, 2000).

Plasmids and Cell Transfection

Full-length (nucleotides 1–1287) and truncated (nucleotides 1–1233) *apm1* cDNAs, encoding μ1 and μ1ΔCt, respectively, were amplified by PCR, sequenced (MWG-Biotech, Ebersberg, Germany), and cloned into the *Dictyostelium* expression vector pDXA-3C (Manstein *et al.*, 1995). Plasmid were transfected in *Dictyostelium* by electroporation as described (Cornillon *et al.*, 2000). γ-Adaptin encoding cDNA was isolated by PCR screening of a cDNA library kindly provided by Dr. Fuller (University of California, San Diego, CA) with oligonucleotides derived from a partial gene sequence (contig6354) encoding part of *Dictyostelium* γ-adaptin. A full-length cDNA insert (GenBank accession number AY144597) was sequenced on both strands and cloned into pDXA-3C. For GFP tagging of γ-adaptin, a GFP insert was obtained by PCR using pTX-GFP as template (Levi *et al.*, 2000) and inserted before the stop coding of γ cDNA. γ-GFP was included in AP-1 complexes and partitioned into membranes and cytosol as native γ-adaptin and, very few monomeric γ-GFP proteins were detected in cytosol fractionated by gel filtration on a Sephacryl S300 column (our unpublished results). GST-γ constructs were made by PCR and subcloned into the vector pGEX-4T (Amersham Pharmacia).

Apm1 Knockout

DNA fragments comprising nucleotides 373–578 (5′ fragment) and 579-1287 (3′ fragment) of *apm1* cDNA were amplified by PCR from DH1-10 genomic DNA and cloned into pCRII-TOPO (Invitrogen, Groningen, Netherlands). The *apm1* knockout construct was made by inserting the blasticidin resistance cassette (Sutoh, 1993) between the 5′ and 3′ *apm1* fragments. The final construct was linearized by *Sal*I and electroporated into DH1-10 cells as for complementation studies. Transformants were selected in the presence of 10 μg/ml blasticidin, and individual colonies were tested by Southern blot and immunoblotting. Four independent clones were identified and displayed identical phenotypes. One clone was chosen for further characterization.

RESULTS

Biochemical Characterization of Dictyostelium AP-1 Complex

Clathrin-associated adaptor complexes are essential components of the mammalian endocytic and biosynthetic transport pathways. As a first step toward the understanding of AP-1 function in *Dictyostelium*, we undertook the molecular characterization of AP-1 subunits in this organism. First, the AP-1 medium chain (μ1) was clearly identified because it shares 69% identity with the μ1A subunit of the mouse AP-1 complex (de Chassey *et al.*, 2001). A rabbit polyclonal antibody against μ1 was raised, and this antiserum recognized a protein with an electrophoretic mobility of 50 kDa (Figure 1A) as predicted from its cDNA sequence. Next, the identification of the γ-adaptin subunit was facilitated by the characterization of an antimurine phagosome mAb (M12A9) that

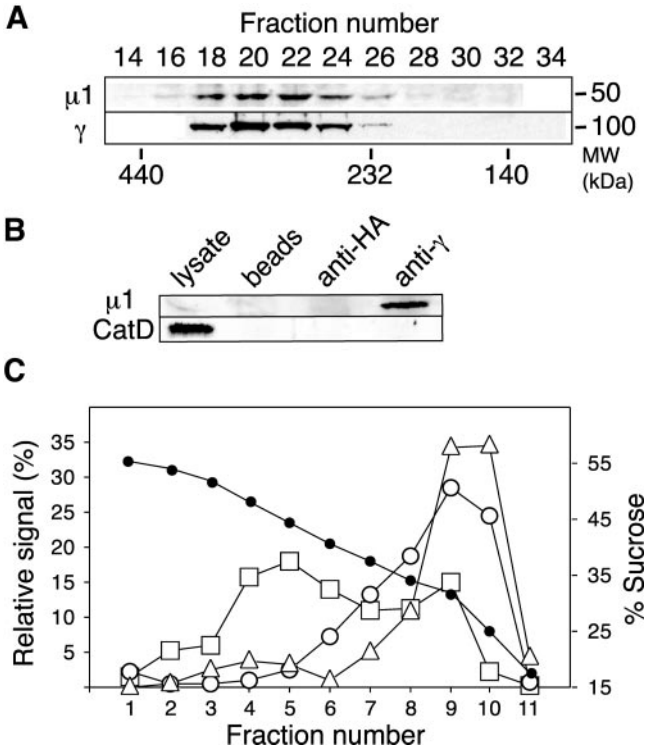

Figure 1. Biochemical characterization of μ1-containing AP complexes. (A) μ1 and γ-adaptin are subunits of 300-kDa cytosolic complexes. *Dictyostelium* cytosol from wild-type (DH1) was fractionated on a Sephacryl S-300 HR gel filtration column. Fractions were collected and proteins were analyzed by Western blotting using the anti-μ1 antiserum or the anti–γ-adaptin mAb M12A9. In DH1 cells, μ1 and γ-adaptin are detected in a peak fraction corresponding to an apparent size of ~300 kDa. (B) μ1 and γ-adaptin belong to same AP-1 complexes. Cell lysates were incubated either with Gammabind beads alone (beads), rat anti-HA antibody bound beads (clone 3F10; anti-HA) or anti–γ-adaptin mAb bound beads (M12A9, anti-γ). Bound proteins were analyzed by immunoblotting using anti-μ1 and anti-Cathepsin D (CatD) rabbit antisera. To evaluate the amount of μ1 and CatD in the lysates, 1/80 of the amount of cells lysate incubated with beads was loaded on the gel (lysate). (C) The μ1 chain is detected in Golgi-enriched fractions. Total membranes were fractionated on linear 15–57% sucrose gradients. After centrifugation at $100,000 \times g$ for 3 h, fractions were collected, and proteins were separated by SDS-PAGE. The distribution of μ1 (circles), comitin (squares), and a Golgi unknown protein (triangles) were determined by Western blotting, quantified, and expressed as a percentage of total signal throughout all fractions. Sucrose percentages are indicated by small closed circles.

cross-reacts with *Dictyostelium* phagosomes (Morrissette *et al.*, 1999). In *Dictyostelium*, this antibody recognized a 100-kDa protein (Figure 1A), consistent with the molecular mass of APs large subunits. Molecular cloning of the corresponding cDNA suggested that this protein could correspond to *Dictyostelium* γ-adaptin because the deduced protein sequence shares 44% of identity with the human γ1-adaptin (Figure 2).

To determine whether μ1 and γ-adaptin associate with other subunits to form a *bona fide* AP-1 complex, *Dictyostelium* cytosol was fractionated by gel filtration on a Sephacryl S300 column. μ1 and γ-adaptin were detected in fractions 16–26, peaking at fraction 20, which corresponds to an apparent size of ~300 kDa (Figure 1A) as observed for AP-1 complexes in different species (Kirchhausen, 1999). The assumption that both chains were subunits of the same adaptor complex was further strengthened by the finding that the μ1 subunit, but not the lysosomal enzyme Cathepsin D (CatD), can be coimmunoprecipitated in cell lysates with the γ-adaptin chain recognized by the anti-γ M12A9 antibody (Figure 1B). Taken together, these results demonstrate the existence of an AP-1 complex in *Dictyostelium.*

Intracellular Localization of the AP-1 Complex in Dictyostelium

In mammalian cells, AP-1 is found not only in the cytosol but also in association with Golgi membranes. To establish the intracellular localization of μ1-containing AP complexes in *Dictyostelium,* membranes were fractionated on linear (15–57%) sucrose gradients (Figure 1C). As previously reported (Bogdanovic *et al.*, 2000), lysosomal compartments were concentrated in high-density fractions (fractions 2–5), whereas plasma membrane, contractile vacuole, and Golgi membranes were contained in low-density fractions (fractions 8–11; unpublished data). Comitin (p24), an actin-binding protein associated with the Golgi apparatus, was previously found in two distinct fractions in sucrose gradients (Weiner *et al.*, 1993). As shown in Figure 1C, μ1 cofractionated with low-density comitin (peaking at fraction 9) in fractions containing Golgi membranes. This result was further confirmed using an antibody (mAb 1/39) against an unknown antigen specifically localized to the Golgi apparatus (Graf *et al.*, 1999). Indeed distribution of μ1 on sucrose gradient was similar to that of the protein detected by mAb 1/39 (Figure 1C). Together, these data indicate that the AP-1 complex is mainly associated with Golgi-enriched fractions in *Dictyostelium*.

The AP-1 localization was next examined by confocal microscopy experiments. Double immunostaining with polyclonal anti–γ-adaptin and anticomitin antibodies revealed that γ-adaptin and comitin (a marker for the Golgi) were mainly concentrated in the same Golgi area as well as in vesicular structures throughout the cytoplasm (Figure 3A). The localization of AP-1 subunits to the Golgi was also confirmed by assessing the distribution of GFP-tagged γ-adaptin (for the biochemical characterization of γ-GFP see MATERIALS AND METHODS). When expressed in *Dictyostelium* cells, γ-GFP localized to the Golgi area labeled with clathrin (Figure 3A), suggesting that in vivo, AP-1 subunits recruit clathrin coats. Some clathrin-coated structures did not colocalize with γ-GFP and could correspond to vesicles including other adaptor proteins. Interestingly, γ-GFP and native γ-adaptin associated with Golgi membranes were insensitive to Brefeldin A (BFA), a fungal metabolite that triggers dissociation of vesicular coats (e.g., COP1 and AP-1) from membranes of mammalian cells (Donaldson *et al.*, 1990; Robinson and Kreis, 1992; Wong and Brodsky, 1992). This lack of sensitivity to BFA was already reported for β-COP in *Dictyostelium* cells and is likely due to the presence of a BFA resistance gene (Mohrs *et al.*, 2000).

Disruption of the actin network using DMSO leads to the reversible fragmentation of the Golgi apparatus and the redistribution of Golgi markers (e.g., β-COP) in the cytoplasm of mammalian and *Dictyostelium* cells (Weiner *et al.*, 1993; Mohrs *et al.*, 2000). To determine whether γ-GFP redistributes as endogenous γ-adaptin during DMSO treatment in vivo, γ-GFP–expressing cells were incubated in 5% DMSO for 0, 20, and 40 min, stained with a polyclonal anti–γ-adaptin antibody, and analyzed by epifluorescence microscopy (Figure 3B). After 20 min of treatment, both γ-GFP and endogenous γ-adaptin showed a diffuse cytosolic stain and also localized to many vesicular structures. However, after 40 min, both proteins reassembled in a perinuclear region with identical kinetics. These results demonstrate that γ-GFP and native γ-adaptin are localized to the same organelle and behave identically when cells are treated with an agent that affects Golgi integrity.

Dictyostelium AP-1 Recruits Clathrin

The colocalization of γ-GFP with clathrin suggested that AP-1 recruits clathrin coats in living cells. To assess clathrin binding of *Dictyostelium* γ-adaptin, we constructed GST-γ fusion proteins and assayed their ability to bind clathrin from *Dictyostelium* cytosol. Analysis of the protein sequence of *Dictyostelium* γ-adaptin revealed only one putative copy of the clathrin box motif, at position 630 (Figure 2), identified in mammalian γ chain as responsible for clathrin binding (ter Haar *et al.*, 2000; Doray and Kornfeld, 2001). Both GST-γ 592–896 (hinge and appendage, with the clathrin box) and GST-γ 663–896 (appendage, without the clathrin box) displayed clathrin binding capacities (Figure 3C). As the presence of the hinge domain resulted in an enhanced recruitment of clathrin in pull-down experiments, the hinge and appendage domains could present independent clathrin binding domains as previously reported for mammalian γ chain (Doray and Kornfeld, 2001).

Intracellular Dynamics of γ-GFP in Living Cells

To determine the dynamic localization of AP-1 in living cells, we observed the intracellular dynamics of γ-GFP–expressing cells by time-lapse confocal microscopy. The main event was the rapid movement of AP-1 structures emerging from the Golgi area toward the cell periphery. These AP-1 structures included vesicles but also tubules and/or queues of vesicles along a same track (Figure 4A). Most AP-1 structures rapidly disappeared when reaching their destination (or were moving out the confocal plane), but in some cases, AP-1 vesicles moved back to the Golgi following the same track (Figure 4B). Together these observations are consistent with the role of AP-1 in the transport of cargo proteins from the TGN to endosomes. In addition, AP-1 structures moving toward the TGN were also observed but at a very low frequency (our unpublished results), al-

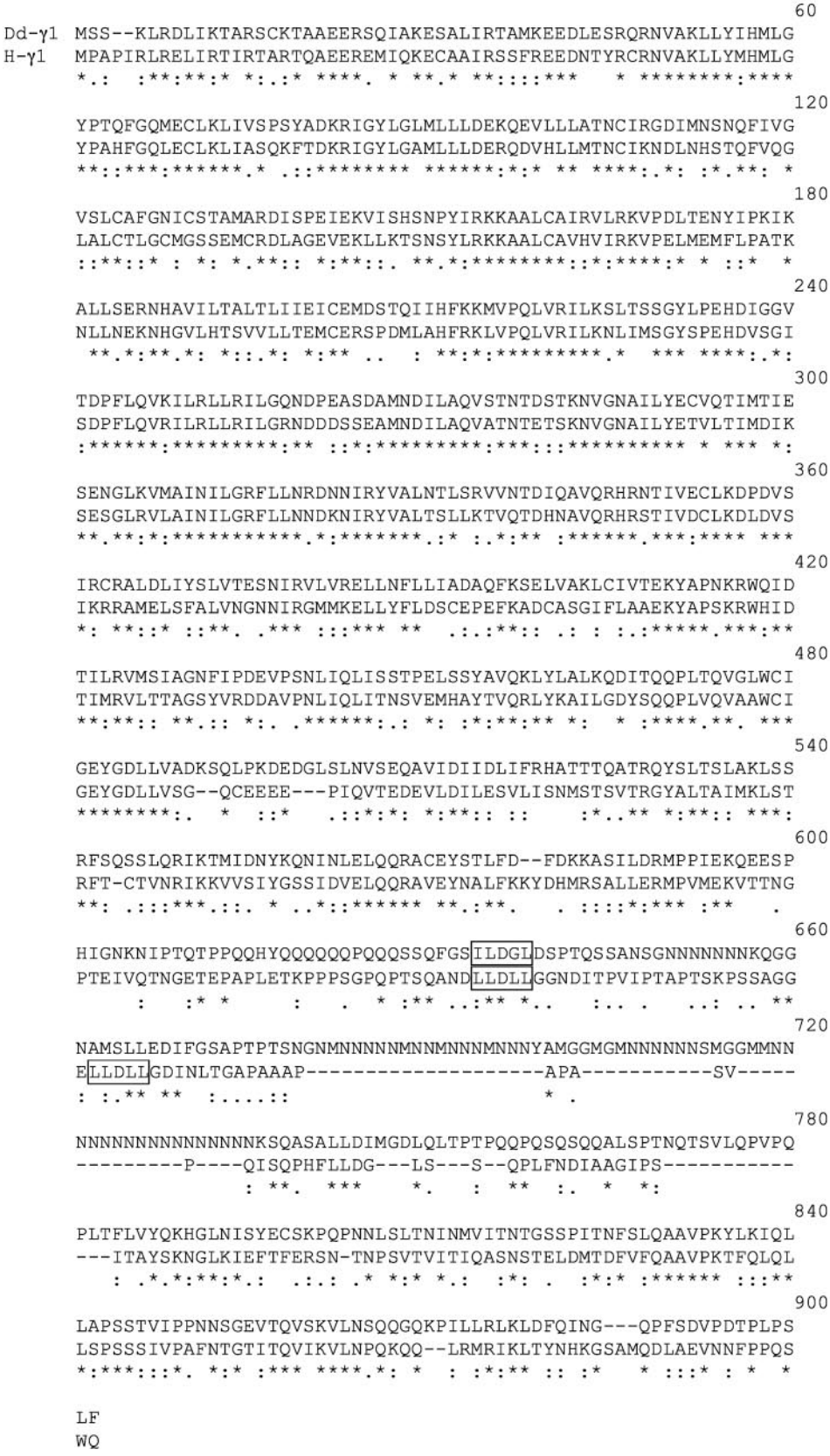

Figure 2. Protein sequence alignment of human (H-γ1) and *Dictyostelium* (Dd-γ1) γ-adaptin chains. Protein sequences were aligned with the Clustal W program available at the www.ibcp.fr web site. Dashes (-) indicate spaces introduced for optimal alignment. Sequences identities (*), strong (:), and weak (.) similarities are indicated below. Boxes show known and putative clathrin binding sequences.

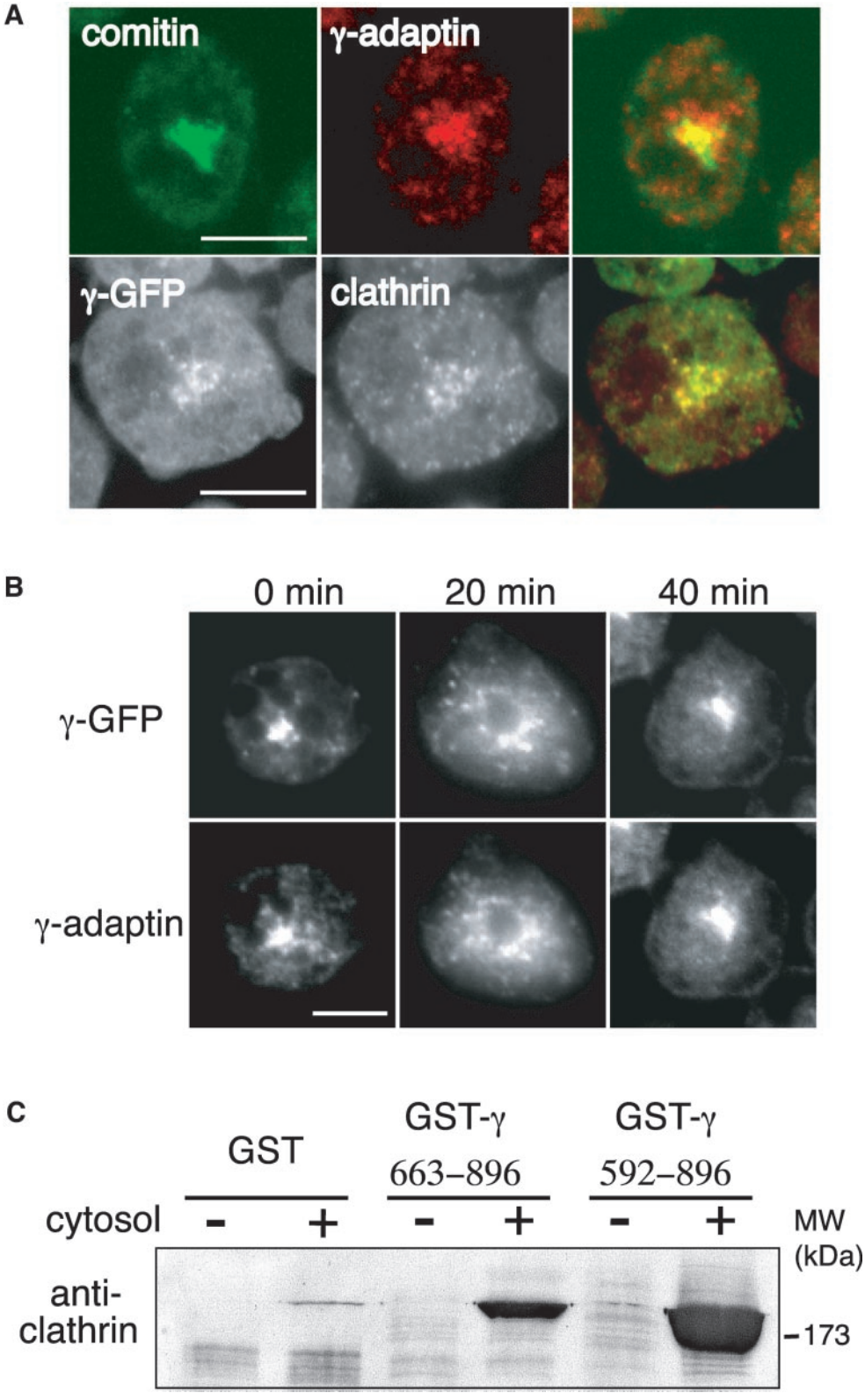

Figure 3. Localization, DMSO sensitivity, and clathrin binding capacity of *Dictyostelium* γ chain. (A) Top: fixed *Dictyostelium* cells were double-labeled with rabbit polyclonal anti–γ-adaptin and mouse anticomitin (190-68-1) antibodies and then incubated with AlexaFluor 488 anti-mouse and Cy3 anti-rabbit secondary antibodies. Confocal microscopy reveals that γ-adaptin and comitin are localized to the same Golgi area. Bottom: *Dictyostelium* cells expressing γ-GFP were fixed, labeled with a rabbit anticlathrin antiserum followed by a Texas red anti-rabbit secondary. The merge picture (right panel) shows γ-GFP (green), clathrin (red), and colocalized structures (yellow) analyzed by confocal microscopy. γ-adaptin mainly colocalizes with clathrin suggesting that in vivo, *Dictyostelium* AP-1 complexes recruit clathrin coats. Bars, 5 μM. (B) *Dictyostelium* cells expressing γ-GFP were incubated in 5% DMSO for the indicated times, fixed, and labeled with rabbit polyclonal anti–γ-adaptin and Cy3 anti-rabbit secondary antibodies. Cells were observed by epifluorescence microscopy. γ-GFP redistributes in cytosol and vacuoles as endogenous γ-adaptin during DMSO treatment. Bars, 5 μM. (C) In vitro binding of clathrin to the hinge and appendage domains of γ. GST, GST-γ (592–896), and GST-γ (663–896) recombinant proteins including the hinge + appendage and appendage domains of γ-adaptin, respectively, were incubated or not (−/+) with cytosol. Bound proteins were separated by SDS-PAGE (7% gel) and revealed by Western blotting using a rabbit polyclonal anticlathrin heavy chain (top panel). Similar amount of GST-γ proteins (twice this amount for control GST) were used in the binding assay. Molecular weights are indicated on the right (kDa).

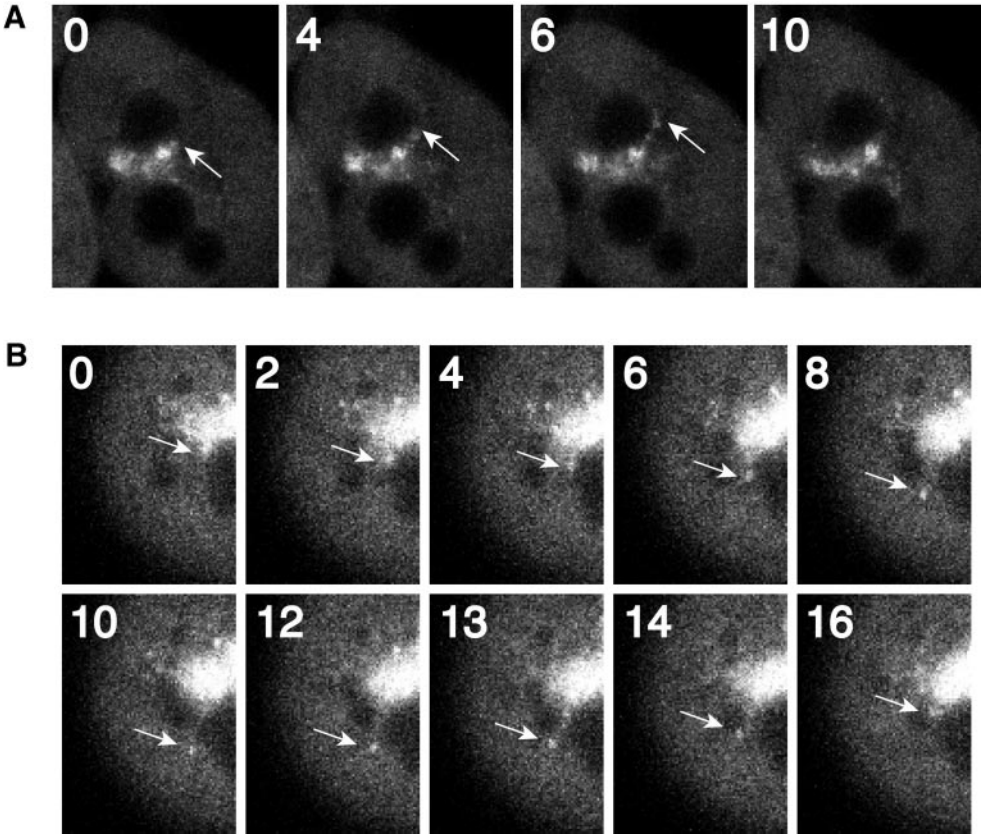

Figure 4. Dynamics of the AP-1 complex in living cells. The mobility of AP-1–containing structures was recorded by time-lapse confocal scanning of cells expressing GFP-tagged γ subunits. (A) The movement of structures containing γ-GFP was recorded during a 10-s time course and revealed vesicles and spherical and tubular structures moving along a same track from the TGN to the cell periphery. (B) The arrow indicates AP-1 structures leaving the Golgi and then moving back to the Golgi following the same track. Times are indicated in seconds in the top left corner of pictures.

though these events were difficult to reliably quantify because AP-1 structures moving along microtubules hardly stayed in a same confocal plane and, consequently, AP-1 structures docking to Golgi membranes could be easily overlooked. This result suggests that AP-1 could be also implicated in retrograde transport between endosomes and the TGN in *Dictyostelium* cells as reported for mammalian and yeast cells.

The μ1 Chain Is Not Required for AP-1 Assembly and Golgi Localization

To determine the role of AP-1 in *Dictyostelium*, we decided to invalidate genes encoding AP-1 subunits. For unknown reasons, we failed to inactivate the γ-adaptin gene, and therefore the *apm1* gene, encoding the μ1 AP-1 subunit, was disrupted by targeted integration of the blasticidin selection marker (see MATERIALS AND METHODS). The μ1 protein was not detected in mutant cells (Figure 5A), whereas stable transfection of native μ1 (*apm1*$^-$ + μ1), or μ1 truncated by deletion of its 18 C-terminal residues (*apm1*$^-$ + μ1ΔCt), restored μ1 expression to levels comparable to wild-type cells (DH1; Figure 5A). The deleted domain in μ1ΔCt corresponds to a region in murine μ2 required for the interaction between μ chains and tyrosine-based signals (Owen and Evans, 1998). This truncated μ1 chain was unable to bind mammalian tyrosine signals in a yeast two-hybrid assay (our unpublished results). Therefore the μ1ΔCt construct was used hereafter to determine whether the observed defects in mutant *apm1*$^-$ cells were due to the absence of a functional μ1 subunit or to any aberrant functions of partial AP-1 complexes deprived of μ1 chains.

To determine the structure of AP-1 complexes in mutant *apm1*$^-$ cells, cytosol was fractionated by gel filtration and fractions were analyzed by immunoblotting with the anti–γ-adaptin antibody. In contrast to wild-type cells, γ-adaptin from *apm1*$^-$ cytosol was detected in fractions 20–26, peaking at fraction 22, which corresponds to an apparent size of ~250 kDa (Figure 5B). Therefore AP-1 subunits are able to assemble without μ1 chains.

A characteristic of AP complexes is to cycle between cytosolic and membrane-bound pools. To evaluate whether AP-1 complexes without μ1 chains partitioned into cytosolic and membrane fractions as native AP-1, we determined the relative amount of γ-adaptin in cytosol (C) and total membrane (M) fractions prepared by centrifugation. In wild-type cells, 87% of total cellular γ-adaptin associated with membranes, whereas only 52% was detected in membranes of *apm1*$^-$ mutant cell (Figure 5C). We next assessed the intracellular localization of AP-1 partial complexes in *apm1*$^-$ cells. As observed in Figure 5D, γ-adaptin was detected in the Golgi area of *apm1*$^-$, *apm1*$^-$ + μ1ΔCt and wild-type cells. Taking together these results demonstrate that μ1 is not essential for specific recruitment of AP-1 to Golgi membranes in *Dictyostelium* cells but participates in membrane binding efficiency.

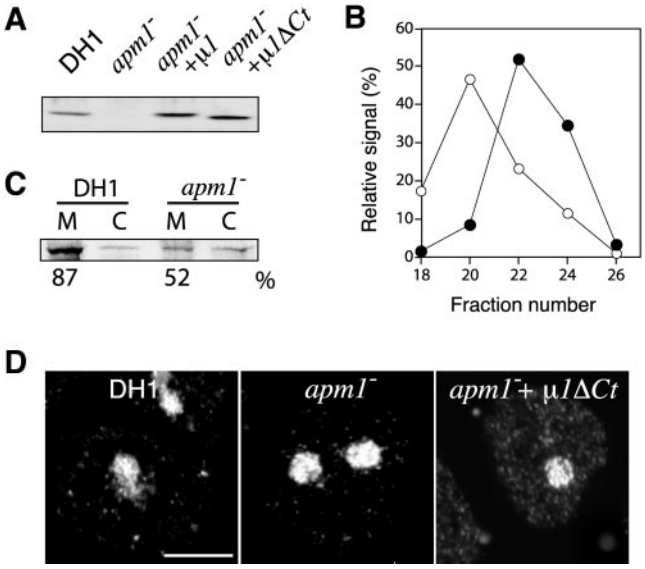

Figure 5. Assembly and Golgi localization of AP-1 without μ1 subunits. (A) Expression of native or mutated forms of μ1 in *apm1*$^-$ cells. Cell lysates from wild-type (DH1), μ1-deleted (*apm1*$^-$), and mutant cells stably transfected with native μ1 (*apm1*$^-$ + μ1) or truncated μ1 (*apm1*$^-$ + μ1ΔCt) were immunoprecipitated with the anti–γ-adaptin antibody and analyzed by Western blotting using the anti-μ1 rabbit antiserum. All transfected cells show comparable levels of μ1 proteins. (B) AP-1 subunits assemble without μ1 chains. *Dictyostelium* cytosol from wild-type (○) and *apm1*$^-$ cells (●) was fractionated on a Sephacryl S-300 HR gel filtration column. Fractions were collected and proteins were analyzed by Western blotting using the anti–γ-adaptin mAb M12A9. In mutant *apm1*$^-$ cells, γ-adaptin was detected in fractions 20–26, peaking at fraction 22, which corresponds to an apparent size of ~250 kDa. (C) Analysis of the association of γ-adaptin with membranes. The indicated cell lines were lysed in breaking buffer (10 mM HEPES, pH7; 200 mM sucrose; 0.5 mM $MgCl_2$; 15 mM KCl; 1 mM EDTA; 1 mM DTT) as described in MATERIALS AND METHODS. Membranes (M) and cytosol (C) were separated by centrifugation at 100,000 × *g* for 1 h. The presence of γ was detected by Western blotting using the anti–γ-adaptin mAb M12A9, quantified, and expressed as the percentage of total γ-adaptin in both fractions. AP-1 complexes without μ1 chains show a reduced binding to membrane compared with native AP-1. (D) Intracellular localization of γ-adaptin in *apm1*$^-$ cells. The indicated cell lines were fixed, incubated with the anti–γ-adaptin polyclonal rabbit antiserum, and stained with a Cy3 anti-rabbit secondary antibody. Bar, 5 μM

apm1 Disruption Causes Defects in Cell Growth and Development

Mutant *apm1*$^-$ cells were viable but grew poorly on bacterial lawns (Figure 6A) and in liquid culture (Figure 6B). Expression of native μ1, but not of μ1ΔCt, in *apm1*$^-$ cells reverted the growth defect. In old cultures, some giant cells with 3–5 nuclei per cell appeared, suggesting cytokinesis defects in this mutant.

On nutrient depletion, *Dictyostelium* cells aggregate and proceed to a developmental cycle leading to the formation of fruiting bodies. Previous studies on clathrin heavy-chain null mutants (chc$^-$) revealed the important role played by clathrin during *Dictyostelium* development (Niswonger and O'Halloran, 1997). chc$^-$ cells are delayed in early develop-

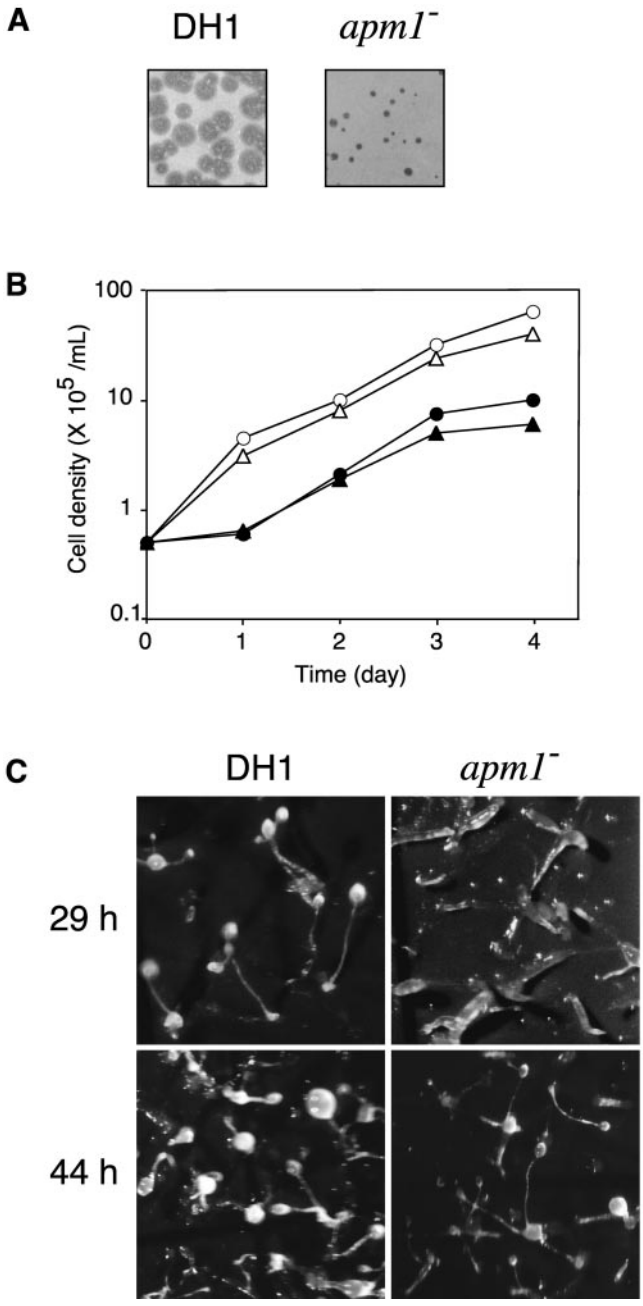

Figure 6. Growth and development of *apm1*$^-$ cells. (A) *apm1*$^-$ cells have a growth defect when fed upon bacteria. To assess growth on bacteria, cells were plated onto *Klebsiella aerogenes* lawns and *Dictyostelium* colonies were observed 7 d later. (B) Cells were grown axenically at 22°C without shaking. Growth was measured by cell counting using a hemocytometer. *apm1*$^-$ mutant (●) and *apm1*$^-$ + μ1ΔCt (▲) cells grow slower than wild-type (○) and *apm1*$^-$ + μ1 (△) cells. (C) The kinetics of development of the *apm1*$^-$ mutant is delayed. For development analysis, cells were plated on 0.45-μm membrane filters laid on Na/K phosphate buffer and incubated at 24°C in humid chambers for the indicated periods of time. Although after 29 h the development of wild-type cells was complete, *apm1*$^-$ cells showed finger-like structures. After 44 h of development, fruiting body-like structures appeared, though the stalks were very thin in comparison to wild-type structures.

ment and fail to produce any spores. To evaluate the role of AP-1 in development, mutant cells were layered onto filters soaked with Na/K phosphate buffer. In contrast to chc$^-$ cells, the early stages of development in *apm1*$^-$ cells (e.g., aggregation) were normal as well as the morphology of each developmental step (our unpublished results). However, the overall kinetics of development was delayed (Figure 6C). After 29 h, although development of wild-type cells was complete, *apm1*$^-$ cells showed finger-like structures. After 44 h of development, fruiting body-like structures appeared, though the stalks were very thin in comparison to wild-type structures. Interestingly, in HL5 medium, only 10% of mutant spores germinated and gave rise to amoeba compared with 70% for control spores. Stable transfection of native μ1 complemented both growth and developmental defects. However, μ1 truncated of the 18 C-terminal residues (μ1ΔCt) did not restore these functions (our unpublished results).

Thus, in *Dictyostelium*, AP-1 seems to be important for cell growth, development, and spore germination. Notably, the μ1ΔCt did not rescue any defects establishing that the absence of a fully functional μ1 chain accounts for the observed phenotypes in *apm1*$^-$ cells.

Lysosomal Enzyme Precursors Are Secreted in apm1$^-$ Cells

In mammalian cells, AP-1 is required for the transport of newly synthesized lysosomal enzymes between the TGN and lysosomes. Accordingly, defects in lysosomal enzymes transport lead to secretion of enzyme precursor forms into the culture medium (Le Borgne and Hoflack, 1998). To test if AP-1 could play a similar role in *Dictyostelium*, we analyzed whether the precursor forms of two lysosomal enzymes (α-mannosidase and cathepsin D) could be detected in culture medium from *apm1*$^-$ mutant cells.

Dictyostelium α-mannosidase is synthesized as a 140-kDa precursor, which, upon transport to lysosomes, is processed into peptides of 60 and 58 kDa (Mierendorf *et al.*, 1983, 1985; Cardelli *et al.*, 1986). In wild-type cells, no precursor form (pro) was detected in the culture medium after 10 h of incubation (Figure 7A) and most of the enzyme in the intracellular fraction (P) was found in its mature lysosomal form (mat). In contrast, in *apm1*$^-$ cells, the α-mannosidase precursor was detected in the culture medium (48% of total enzyme secreted after 10 h) and less mature form (mat) accumulated relative to the amount of secreted precursor (Figure 7A). Complementation of mutant cells with native μ1, but not with μ1ΔCt (38% of enzyme secreted after 10 h), reverted this phenotype, although more intracellular mature form was detected in *apm1*$^-$ + μ1ΔCt than in *apm1*$^-$ cells, suggesting that μ1ΔCt could partially restore AP-1 sorting functions. The fate of cathepsin D, another lysosomal enzyme, was also assayed and identical maturation and secretion defects were observed (our unpublished results).

These results demonstrate that AP-1 is required for the targeting of two lysosomal enzymes to lysosomes, indicating that *Dictyostelium* and mammalian AP-1 adaptor complexes share similar functions. To exclude the possibility that *apm1*$^-$ mutant cells display a general membrane trafficking defect, we analyzed the internalization rate of a cell surface transmembrane protein (p80) known to be constitutively internalized (Ravanel *et al.*, 2001). Wild-type cells showed ~80% of p80 endocytosis after 30 min monitored by flow cytometry, and the rate of endocytosis was similar in *apm1*$^-$ cells (our unpublished results), excluding a major defect in membrane protein endocytosis in mutant cells.

Endocytic Compartments in apm1$^-$ Cells

As the transport of lysosomal components is impaired in *apm1*$^-$ cells, the *apm1*$^-$ deletion could also affect the overall biogenesis and/or morphology of the endocytic compartments. To assess this possibility, we analyzed the intracellular distribution of a transmembrane endosomal protein, p80, in wild-type and mutant cells. The p80 protein is present at the cell surface but also throughout the endocytic pathway. As previously described (Ravanel *et al.*, 2001), in wild-type cells, p80 was detected in early endocytic compartments (characterized by a low concentration of p80) and also in late endocytic compartments (characterized by a high concentration of p80; Figure 7B). In *apm1*$^-$ cells, p80 was similarly distributed in endocytic compartments with high and low p80 content. However, although this marker was normal in location, the size of these endocytic compartments appeared smaller and the number of vacuole structures was significantly increased (Figure 7B). To demonstrate that these morphological defects were not restricted to p80-containing vacuoles, cells were allowed to internalize the fluid-phase marker FITC-dextran for 60 min to label all endocytic compartments and analyzed by epifluorescence microscopy. In these conditions, identical defects in size and number of vacuoles were observed and the expression of μ1ΔCt in *apm1*$^-$ cells did not restore the wild-type phenotype (Figure 7C). Electron microscopy analysis further confirmed these results. In contrast to wild-type cells that displayed several large vacuoles, *apm1*$^-$ cells displayed only one or two medium-size vacuoles surrounded by many small vacuoles (Figure 8). Finally the localization of two other endosomal markers, the actin binding protein coronin (early and late endosomes) and the coat protein vacuolin (postlysosomal vacuoles), was also found identical in *apm1*$^-$ and wild-type cells (Figure 7B and our unpublished results). Therefore, *apm1*$^-$ mutant cells present a normal organization of the endocytic pathway but an altered morphology of endocytic organelles.

AP-1 Is Required for Transport of Proteins to the Contractile Vacuole Complex

To live in hypo-osmotic condition, *Dictyostelium* cells have developed a specialized compartment, the contractile vacuole complex (CV). This osmoregulatory organelle is composed of large cisternae (bladder) and interconnecting ducts. The cisternae fuse periodically with the plasma membrane to expel water from the cells. Because clathrin-deficient cells display osmoregulatory defects and the CV complex is missing (O' Halloran and Anderson, 1992), we first assayed the function of the CV complex in *apm1*$^-$ mutant cells. Wild-type and mutant cells were shifted from culture medium to water and observed >30 min. Rapidly, both cell types swelled and rounded up because of water import. Although wild-type cells rapidly adjusted to the osmotic stress and returned to their original shape, *apm1*$^-$ mutant cells stayed swollen (Figure 9). The expression of native μ1, but not of μ1ΔCt, allowed *apm1*$^-$ mutant cells to regain the ability to adjust to osmotic stress (Figure 9).

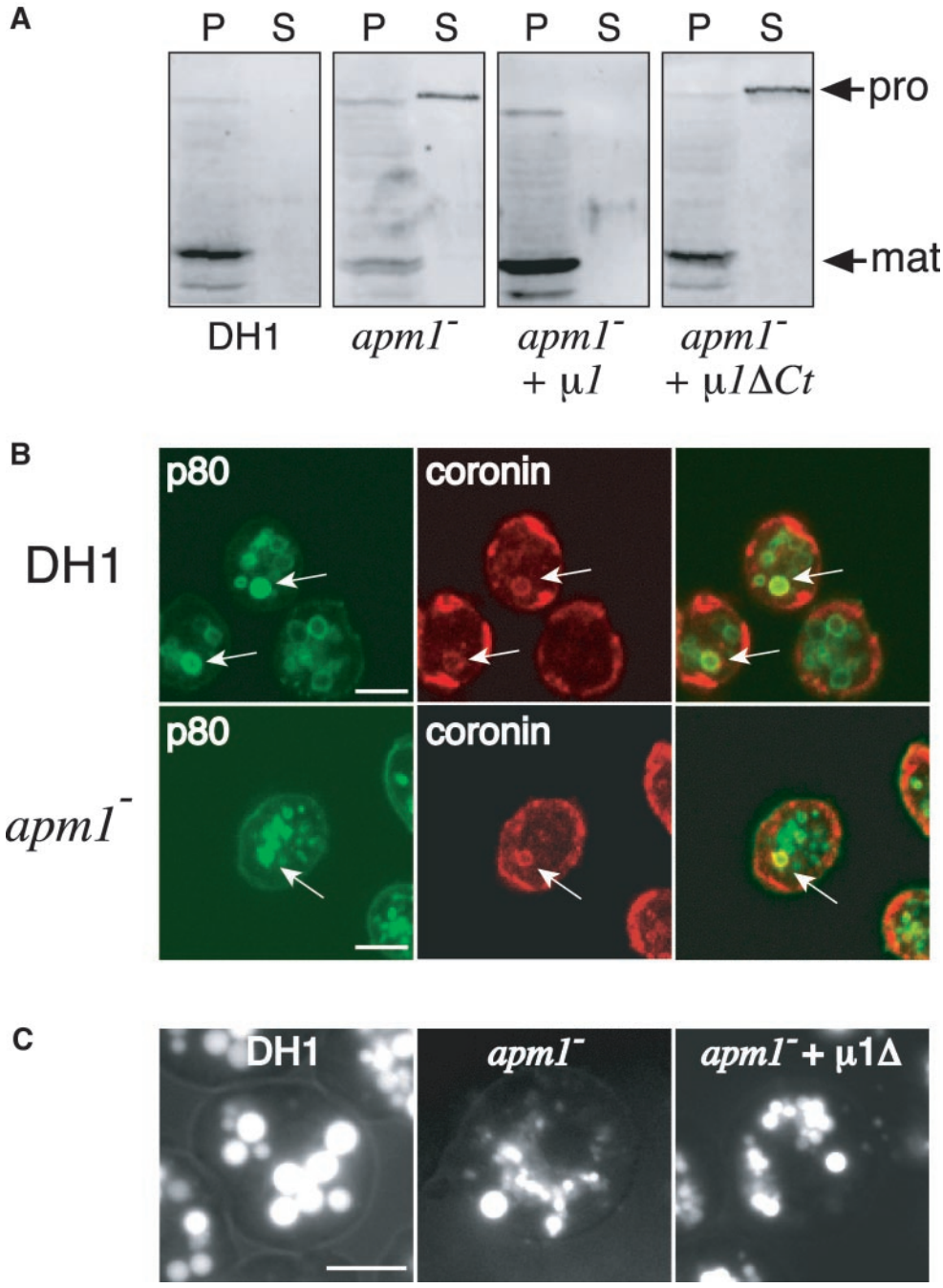

Figure 7. The transport of lysosomal enzymes is defective in *apm1*$^-$ cells. (A) Secretion of the α-mannosidase immature precursor. Wild-type (DH1), *apm1*$^-$ mutant (*apm1*$^-$), and mutant cells stably transfected with native μ1 (*apm1*$^-$ + μ1) or truncated μ1 (*apm1*$^-$ + μ1ΔCt) were resuspended in HL5 at 5×10^6 cells/ml and incubated at 22°C on a rotating wheel. After 10 h, cells were centrifuged, and supernatants (S) and cell pellets (P) were analyzed by Western blotting. In wild-type cells, α-mannosidase is synthesized as a 140-kDa precursor (pro) which, when transported to lysosomes, is processed into peptides of 60 and 58 kDa (mat) not separated on this gel. Although no precursor is secreted in DH1 or *apm1*$^-$ + μ1 cells, the α-mannosidase precursor was secreted in *apm1*$^-$ and *apm1*$^-$ + μ1ΔCt cells. (B) p80 is correctly distributed in endocytic compartments of *apm1*$^-$ cells. Methanol fixed wild-type (DH1), *apm1*$^-$ mutant (*apm1*$^-$) cells were stained with an anticoronin antibody (176-2-5), followed by an Alexa Fluor 568 antimouse secondary antibody and finally incubated with the anti-p80 antibody (H161) directly coupled to Alexa Fluor 488. Arrows indicate vacuoles with high content of p80 and displaying coronin. p80 and coronin are correctly distributed in all endocytic compartments. However, the size of these compartments appears smaller and the number of vacuole structures is significantly increased in *apm1*$^-$ mutant cells. Bars, 5 μM. (C) The morphological defects are not restricted to p80 containing vacuoles. Cells grown on coverslips were allowed to internalized the fluid-phase marker FITC-dextran for 60 min to labeled all endocytic compartments and analyzed by epifluorescence microscopy after overlay with a thin layer of an agarose. In these conditions, *apm1*$^-$ and *apm1*$^-$ + μ1ΔCt cells show identical defects in the size and the number of vacuoles. Bars, 5 μM.

Next we examined the integrity of the CV complex by studying the localization of two CV resident proteins, Rh50 (*Dictyostelium* rhesus protein; Benghezal *et al.*, 2001) and VatA (a subunit of the peripheral vacuolar-H^+-ATPase V1 domain; Zhu and Clarke, 1992; Jenne *et al.*, 1998; Neuhaus *et al.*, 1998; de Hostos, 1999) in *apm1*$^-$ mutant cells. In wild-type and *apm1*$^-$ + μ1 cells, Rh50 localized exclusively to the CV network, whereas VatA was mainly present in the CV network but also was detected in early endosomes. In contrast, Rh50 and VatA did not colocalized to the CV network in *apm1*$^-$ and *apm1*$^-$ + μ1ΔCt mutant cells (Figure 10A). Instead, Rh50 was mainly found on punctuate structures in the Golgi area stained with the anticomitin antibody, whereas VatA was mainly detected in vacuoles, which were likely endosomes because they contained p80 (Figure 10B). In addition, Calmodulin, another CV component (Ultricht and Soldati, 1999), also redistributed in endosomes in *apm1*$^-$ cells, and the characteristic CV network was not detected in *apm1*$^-$ mutant cells incubated with FM 4–64 (a fluorescent dye known to label the CV complex; our unpublished results). Thus, no recognizable CV network can be detected in *apm1*$^-$ cells, although it is not clear whether Rh50-

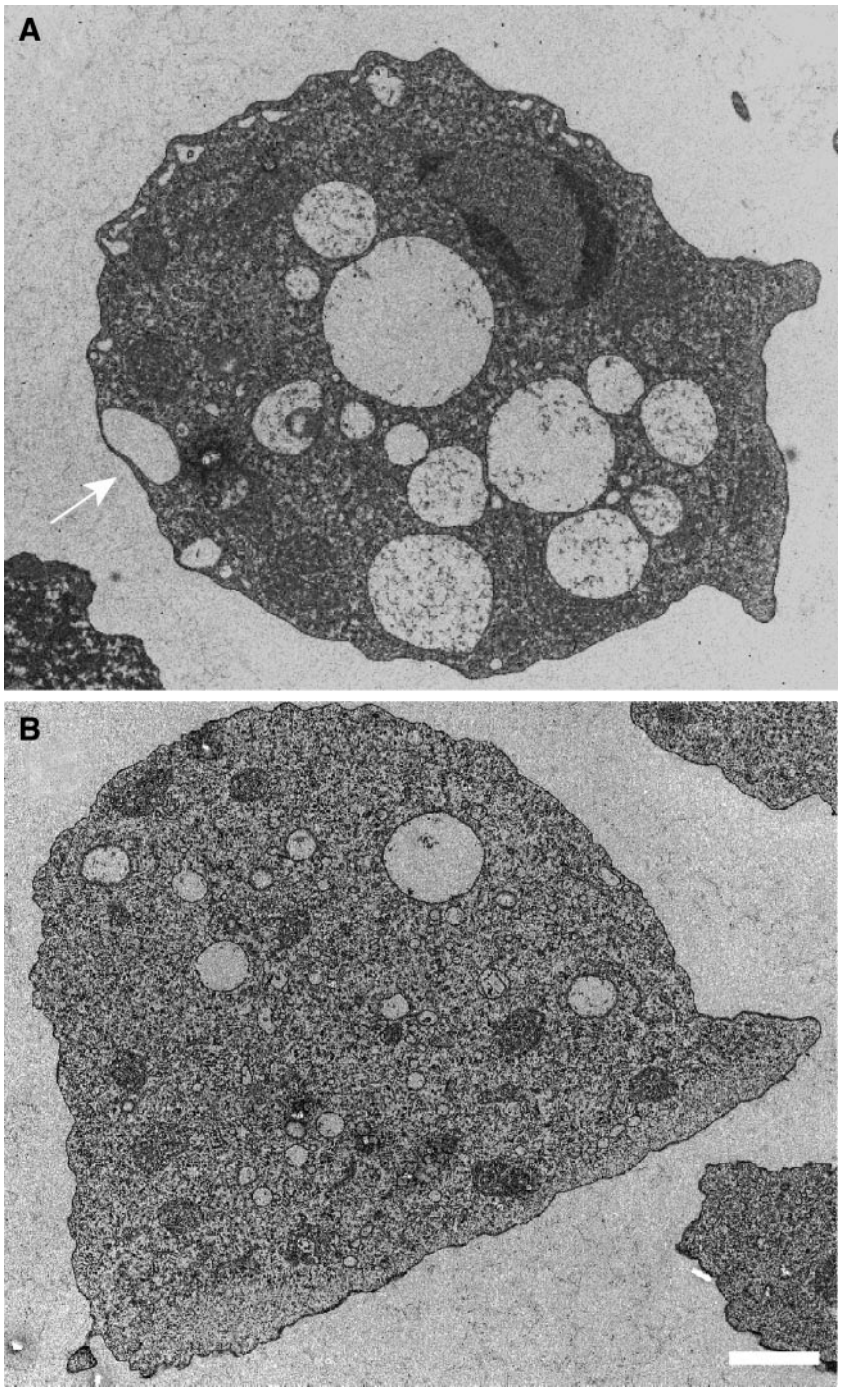

Figure 8. Ultrastructure of endocytic compartments in mutant *apm1*$^-$ cells. Thin sections electron microscopy analysis of wild-type (A) and *apm1*$^-$ (B) cells reveals that mutant cells display only one or two medium-size vacuoles surrounded by small vacuoles in contrast to wild-type cells containing several large vacuoles. The arrow indicates a translucent vacuole likely to correspond to a contractile vacuole. Bars, 1 μM

positive structures correspond to altered nonfunctional contractile vacuoles or to mislocalized Rh50, for instance to the Golgi apparatus.

The absence of functional CV and the mis-localization of CV components suggested that the morphology of the CV complexes was altered in *apm1*$^-$ mutant cells. In electron microscopy images, CV appears as large translucent vacuoles (Chastellier *et al.*, 1978; Quiviger *et al.*, 1978). Electron microscopy analysis of thin section series of *apm1*$^-$ mutant cells did not reveal the presence of large vacuoles characteristic of CV organelles (Figure 8).

DISCUSSION

In this study, we report the function of a clathrin-associated complex in the model organism *Dictyostelium discoideum*. We

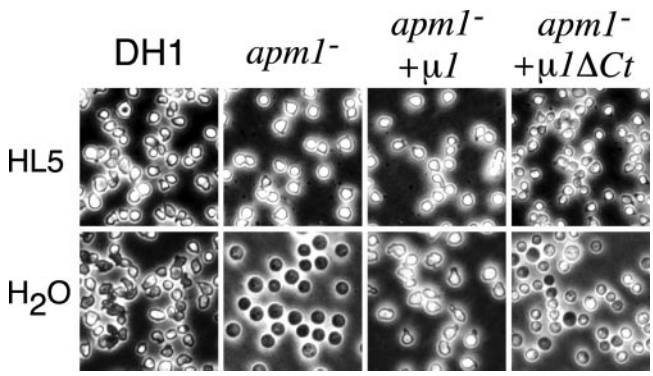

Figure 9. Mutant *apm1*$^-$ cells display osmoregulatory defects. Wild-type (DH1) and mutant cells were shifted from culture medium (HL5) to water and observed after 30 min by contrast-phase microscopy. Rapidly, both cell types swelled and rounded up because of water import. While wild-type cells rapidly adjusted to the osmotic stress and returned to their original shape, *apm1*$^-$ mutant cells stayed swollen because of water import. The expression of native μ1 (*apm1*$^-$ + μ1) but not of μ1ΔCt (*apm1*$^-$ + μ1ΔCt) allows *apm1*$^-$ mutant cells to regain their normal shape.

focused on AP-1 which, in mammalian cells, is most implicated in transport from the TGN to endocytic compartments. We made the molecular characterization of *Dictyostelium* AP-1 and showed that this complex displays similar composition, localization, and dynamics than mammalian AP-1 complexes. Taking advantage of *Dictyostelium* genetic tools, we demonstrate for the first time that the deletion of one single AP subunit, μ1, results in strong phenotypes, including defects in basic cell functions and in several clathrin-dependent transport pathways. We provide the first in vivo evidence that μ1 Ct domain, which interacts with tyrosine-based sorting signals, is essential for the correct targeting of lysosomal hydrolases from the Golgi compartment to lysosomes. Furthermore, we demonstrate that the AP-1 coat is implicated in the biogenesis of a functional osmoregulatory organelle, the contractile vacuole.

Molecular Characterization and Dynamics of Dictyostelium AP-1

The high homology of γ-adaptin and μ1 chains with corresponding mammalian AP-1 subunits strongly suggested that these chains were subunits of a same AP-1 complex. This assumption was confirmed by showing that these chains assemble with putative β1 and σ1 subunits to form in a high-molecular-weight complex of ~300 kDa, consistent with the size of an heterotetrameric AP complex (Figure 1A). Although these β1 and σ1 chains were not characterized in this study, blast searches in available EST and genomic data basis indicate potential genes encoding β1 (contig11712 encoding a protein of 927 aa sharing 52% identify and 28% similarity with mouse β1) and σ1 subunits (cDNA clone SSF227 encoding a deduced protein sequence of 154 aa sharing 55% identity and 28% similarity with mouse σ1).

The identification of this AP complex as *Dictyostelium* AP-1 was further established by studying its intracellular localization. Subcellular fractionation studies indicate that the AP-1 complex is mainly associated with Golgi-enriched fractions (Figure 1C). Confocal microscopy analyses show that AP-1 is concentrated in the Golgi area (Figure 3A) as well as vesicular structures. Finally, a γ-GFP fusion protein expressed in *Dictyostelium* cells localizes to this same perinuclear region (Figure 3A), which undergoes a reversible fragmentation when cells are treated with DMSO, an agent that affects Golgi integrity (Figure 3B). Therefore the intracellular localization of AP-1 in *Dictyostelium* is similar to that observed for mammalian AP-1 complexes (Robinson, 1989).

In mammalian and yeast cells, AP-1 has been reported to be involved in retrograde (endosomes to TGN) transport of several proteins (Zizioli *et al.*, 1999; Meyer *et al.*, 2000; Valdivia *et al.*, 2002). However, live microscopy studies of cells expressing YFP-tagged-μ1A chains indicates that AP-1–coated structure moving toward the TGN are hardly observed (Huang *et al.*, 2001), thus leaving open the debate on the transport pathway in which operate AP-1 complexes. The incorporation of a functional γ-GFP protein into AP-1 complexes allowed us to examine the dynamics of the AP-1 coat in *Dictyostelium* living cells. As described in mammalian cells (Huang *et al.*, 2001), AP-1 labeled with γ-GFP is detected in vesicles, spherical and tubular structures moving along a same track from the TGN to the cell periphery and very rarely toward the TGN. Therefore the main function of AP-1 in *Dictyostelium* appears to be in anterograde (TGN to endosomes) transport. Surprisingly, we also detected vesicles moving back and forth between TGN and endosomes. This observation suggests that AP-1 vesicles could rapidly deliver cargo proteins to endosomal compartments without fusion between vesicles and endosomes and the loss of vesicles integrity. Alternatively, this movement could reflect the Golgi dynamics, which has been reported to varied between a compact organization and a dispersed structure accompanied by fast protrusions of Golgi tubules and vesicles (Schneider *et al.*, 2000). Further studies will be required to test these hypotheses.

An essential feature of AP-1 complexes in all species is to facilitate the recruitment of cytosolic clathrin onto nascent vesicles. This process occurs through a direct interaction between clathrin and sequences in the hinge domains of β1 and γ-adaptin (Shih *et al.*, 1995; ter Haar *et al.*, 2000; Doray and Kornfeld, 2001). As expected, immunofluorescence microscopy studies in *Dictyostelium* cells establish that γ-adaptin mainly localizes to clathrin-coated vesicles (Figure 3A). Furthermore, GST-γ fusion proteins comprised of either the hinge and appendage domains or only the appendage region displayed clathrin-binding abilities (Figure 3C) as reported for mammalian γ subunit of AP-1 (Doray and Kornfeld, 2001). Taken together, these results support the conclusion that we have identified a bona fide AP-1 complex in *Dictyostelium* with features comparable to that of mammalian AP-1.

Dictyostelium, A Model Organism To Study the Function of AP-1

Yeast genetic studies aimed at understanding the function of AP-1 have been hindered by the absence of major trafficking defects in μ1-deleted strains (Stepp *et al.*, 1995). On the contrary, deletions of μ1A and γ chains in mice lead to embryonic lethality, also preventing functional analysis of AP-1 in vivo (Zizioli *et al.*, 1999; Meyer *et al.*, 2000). To overcome this problem, we took advantage of the molecular

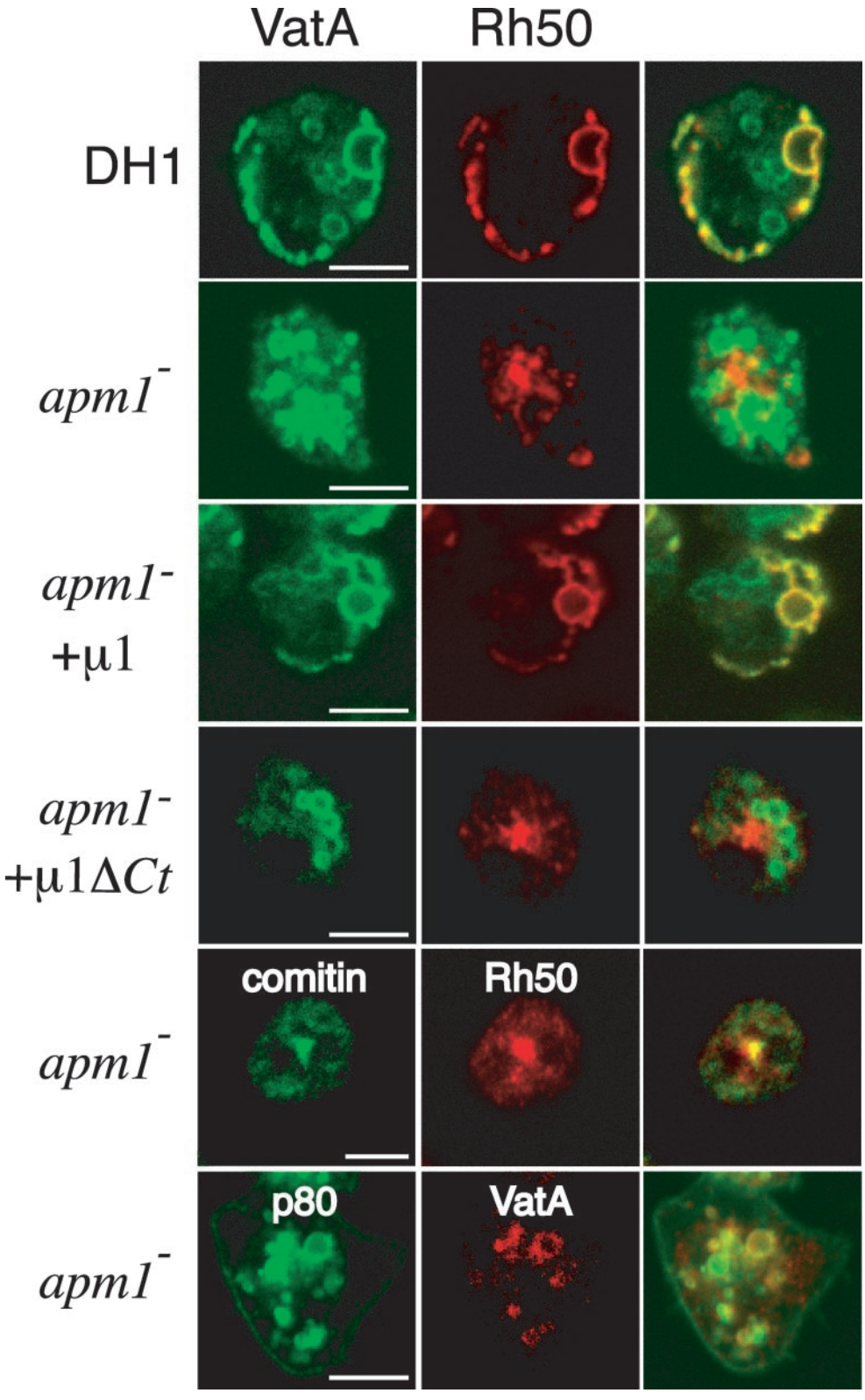

Figure 10. AP-1 is required for transport of CV components. (A) The localization of two CV resident proteins, Rh50 and VatA (a subunit of the peripheral vacuolar-H^+-ATPase V1 domain), was analyzed in the indicated cell lines. Cells were double-labeled with anti-Rh 50 (rabbit antiserum) and anti-VatA (mAb 221-35-2) antibodies, followed by Alexa Fluor 488 anti-mouse and Cy3 anti-rabbit secondary antibodies. In wild-type and *apm1*$^-$ + μ1 cells, Rh50 and VatA colocalize to the CV, whereas in *apm1*$^-$ and *apm1*$^-$ + μ1ΔCt cells, Rh50 and VatA are not detected in the CV network. (B) *apm1*$^-$ mutant cells were double-labeled with combinations of the indicated antibodies. Anti-Rh 50 (rabbit polyclonal), anti-VatA (mAb 221-35-2), anti-p80 (H161 coupled to Alexa Fluor 488), and anticomitin (mAb 190-68-1) followed by the appropriate secondary antibodies (Alexa Fluor 488 anti-mouse, Alexa Fluor 568 anti-mouse, and Cy3 anti-rabbit antibodies). In contrast to wild-type cells, in *apm1*$^-$cells Rh50 mainly colocalizes with comitin and VatA is detected in p80-containing endosomes. Bars, 5 μM.

characterization of AP-1 in *Dictyostelium* and of the molecular genetic techniques well developed in this model organism. The *apm1* gene, encoding *Dictyostelium* μ1 chain, was disrupted by inserting a blasticidin resistance marker gene. In contrast to yeast cells and mice, *apm1* deletion results in viable cells with several phenotypic alterations, indicating that *Dictyostelium* is a unique and valuable model to study the function of AP-1 in vivo.

μ1 Is Not Required for AP-1 Subunits Assembly and Membrane Recruitment

In *apm1*$^-$ mutant cells, other AP-1 subunits are still able to form partial AP-1 complexes (Figure 5B), which are correctly localized to the Golgi apparatus (Figure 5D). These results demonstrate that μ1 is dispensable for the specific docking of AP-1 to Golgi membranes. However, these partial complexes are less stably associated with membranes because only 52% of total γ-adaptin are detected in membranes prepared from *apm1*$^-$ mutant cells compared with 87% for wild-type cells (Figure 5C). Because AP μ chains are involved in the recognition of tyrosine-based signals found on transported proteins, our results demonstrate that the recognition of cargo proteins by μ1 chains is not necessary for membrane recruitment of AP-1 in *Dictyostelium* cells but contributes to the efficiency or/and the stability of the interaction.

How is AP-1 deprived of μ1 subunit still targeted to the right compartment? One possibility is that another AP-1 subunit (e.g., β1) could also participate in the recognition of cargo proteins and therefore this interaction could be sufficient for specific binding of partial AP-1 complexes to Golgi membranes. On the other hand, determinants responsible for membrane localization of APs have been identified in α and γ chains of AP-2 and AP-1 complexes, respectively (Page and Robinson, 1995). More recently, AP-2 complexes have been shown to interact with phosphoinositides through two phospholipid binding domains in the α chain and the surface of μ2 Ct domain (Gaidarov and Keen, 1999; Collins *et al.*, 2002; Rohde *et al.*, 2002). Sequence alignment of α and μ2 with AP-1 γ and μ1, respectively, indicates that AP-1 could also bind phospholipids (Collins *et al.*, 2002). Accordingly, the recognition of Golgi specific phospholipids by γ-adaptin could be sufficient for membrane binding of partial AP-1 complexes in *apm1*$^-$ cells. This hypothesis is in agreement with the finding that AP-1 can be recruited in an ARF-1 dependent manner to protein-free soybean liposomes (Zhu *et al.*, 1999). However, the presence of peptides containing tyrosine-based signals in liposomes clearly enhances AP-1 recruitment to soybean liposomes, indicating that cargo proteins are also involved in AP-1 recruitment (Crottet *et al.*, 2002). Our results are in agreement with these in vitro studies because native AP-1 associates with membranes more efficiently than AP-1 complexes without μ1 chains (Figure 5C). Notably, AP-1 complexes deprived of mouse μ1A subunits do not associate with membranes (Meyer *et al.*, 2000) suggesting that in mammalian cells, lipid and cargo interactions through other AP-1 subunits are not sufficient for the recruitment of partial AP-1 complexes to Golgi membranes. This discrepancy between *Dictyostelium* and mammalian AP-1 complexes could reflect their respective affinity for sorting signals and membrane lipids between species.

μ1 Deletion Affects AP-1 and Clathrin Functions

Clathrin heavy chain null mutant in *Dictyostelium* (chc$^-$ mutant) displays pleiotropic defects, including growth and developmental defects, as well as the inhibition of several transport events along the endocytic pathway (O'Halloran and Anderson, 1992; Ruscetti *et al.*, 1994; Niswonger and O'Halloran, 1997). Clathrin is known to be recruited by several proteins at different intracellular locations, preventing thus to analyze the precise role of clathrin-coated structures in any individual transport pathway. Our study allows for the first time to ascribe some defects observed in chc$^-$ mutant cells to the AP-1 adaptor complex.

As reported for chc$^-$ cells, *apm1*$^-$ cells are viable but grow slower than wild-type cells (Figure 6, A and B). In addition, mutant cells show a much weaker defect in *Dictyostelium* development compared with chc$^-$ cells, suggesting that other clathrin-associated coats are involved in *Dictyostelium* development. The fact that *Dictyostelium* AP-4 medium chain is upregulated during development reinforces this possibility (de Chassey *et al.*, 2001).

In *Dictyostelium* as in mammalian cells, clathrin has been implicated in the transport of lysosomal enzymes between the Golgi apparatus and lysosomes. For instance, chc$^-$ mutant cells secrete immature forms of the hydrolase, α-mannosidase (Ruscetti *et al.*, 1994). In *apm1*$^-$ cells, the precursor form of this enzyme is also secreted (Figure 7A), supporting the conclusion that *Dictyostelium* AP-1 is required for the transport of lysosomal enzymes from TGN to lysosomes. However, this defect is only partial because mature α-mannosidase is still detected intracellularly. Cell surface internalization and further processing of the secreted immature precursor is likely to account for this residual mature enzyme as reported for mannose-6-phosphate receptors in μ1A deleted fibroblasts (Meyer *et al.*, 2000). Alternatively, *Dictyostelium* hydrolases could be transported to lysosomes through an AP-1–independent mechanism, for instance through a GGA (Golgi-localized γ-ear–containing Arf-binding proteins) mediated transport pathway as described for several proteins in other organisms (Black and Pelham, 2000; Dell'Angelica *et al.*, 2000; Hirst *et al.*, 2000; Zhdankina *et al.*, 2001).

In mammalian cells, transport of lysosomal enzymes is mediated by MPRs. As MPRs have not been identified in *Dictyostelium*, our results suggest the existence of another lysosomal enzyme receptor whose trafficking is AP-1 dependent. Recently, targeted disruption of the AP-1 μ1A gene in mice has revealed that AP-1 may act in MPRs retrieval from early endosomes to the TGN (Meyer *et al.*, 2000). This retrieval function was also described in yeast, where the disruption of the μ1 gene inhibits Golgi retrieval of chitin synthase III and syntaxin Tlg1p (Valdivia *et al.*, 2002). Our data in *Dictyostelium* are consistent with the role of AP-1 in this endosomes to TGN retrograde pathway, because a block in recycling of MPR like receptor would also lead to a general defect in lysosomal enzyme targeting. However, the Golgi steady state localization of AP-1 and the fact that AP-1–coated structures moving toward the Golgi area are hardly observed suggest that in *Dictyostelium*, AP-1 is mainly involved in sorting putative MPR-like proteins at the TGN level. The reason for this discrepancy is unknown. Studies on other clathrin-dependent transport processes from the TGN could provide missing clues.

The morphology of the endocytic pathway is affected both in *apm1*$^-$ and chc$^-$ cells. Despite of lysosomal enzyme targeting defects, *apm1*$^-$ mutant cells present a normal localization of several other endocytic protein markers (Figure 7B), suggesting that the overall organization of the endocytic pathway is not altered. In contrast, the morphology of the endocytic organelles is modified in *apm1*$^-$ as well as in chc$^-$ cells. Conventional thin-section electron microscopy reveals the absence of large vacuoles in AP-1–deficient cells and a higher density of small vacuoles (Figure 8). Endo-lysosomal organelles are known to undergo repeated homotypic and heteropypic fusion events, leading to the formation of big vacuoles referred to as postlysosomes or secretory lysosomes (Neuhaus *et al.*, 2002). It is tempting to propose that clathrin/AP-1 complexes could be implicated in sorting events essential for the biogenesis of late endocytic compartments. For instance, essential components of the fusion machinery (e.g., SNARES) required for specific fusion events between vacuoles could be incorrectly addressed in the absence of functional AP-1 complexes, thus preventing the formation of large vacuoles. Conversely, AP-1–coated vesicles could contribute to the constant flux of membrane required to establish and maintain the structure of these organelles.

Interestingly, *apm1*$^-$ mutant cells display a reduced rate of fluid-phase endocytosis and phagocytosis (our unpublished results), whereas endocytosis of a plasma membrane receptor is not affected. In chc$^-$ cells, all these endocytic pathways are altered to some extent. Although receptor-mediated endocytosis in *Dictyostelium* is likely to involved AP-2 clathrin associated adaptor complexes, our results suggest that AP-1 could play a role in pinocytosis and phagocytosis. This possibility is currently under investigation.

Finally, the contractile vacuole complex, an osmoregulatory organelle in *Dictyostelium* also involved in Ca^{2+} regulation (Moniakis *et al.*, 1999), is an integral compartment distinct from endosomes and the plasma membrane. Although endosomes and CV share common components (Bush *et al.*, 1994; Temesvari *et al.*, 1996), these two compartments are apparently not connected to each other because no protein and membrane exchanges occur (Gabriel *et al.*, 1999; Clarke *et al.*, 2002). Clathrin-coated vesicles have been proposed to play an essential role in the biogenesis of CV complexes by transporting proteins and membrane required for CV formation (O'Halloran and Anderson, 1992). Here we show for the first time, that AP-1 is involved in the targeting of two CV components. Rh50, a membrane protein exclusively restricted to the CV network (Benghezal *et al.*, 2001), is mostly retained in the Golgi apparatus in AP-1 deficient cells whereas the vacuolar H^+-ATPase (VatA) is routed to endosomes, its other physiological localization (Figure 10). Targeting defects of these CV components correlate with the absence of functional and morphologically detectable CV complexes (Figures 8 and 9). Together these results suggest that AP-1 is implicated in the biogenesis of the CV complex.

In conclusion, our study demonstrates that the genetically tractable model organism, *D. discoideum*, is a valuable model for studying the function of AP vesicular coat proteins in vivo. Further genetic approaches are now being used to identify additional components implicated in the AP-1–dependent transport machinery.

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