Clathrin Heavy Chain Is Required for Pinocytosis, the Presence of Large Vacuoles, and Development in *Dictyostelium*

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Abstract. To investigate the intracellular role of the clathrin heavy chain in living cells, we have used "antisense" RNA to engineer mutant *Dictyostelium discoideum* cells that are severely deficient in clathrin heavy chain expression. Immunoblots stained with an anti-clathrin heavy chain antiserum revealed that mutant cells contained undetectable amounts of clathrin heavy chain protein. Similarly, Northern blots showed an absence of clathrin heavy chain mRNA. Clathrin heavy chain–deficient *Dictyostelium* cells were viable, but exhibited growth rates twofold slower than parental cells. Whereas many morphological features of the mutant cells were normal, mutant cells lacked coated pits and coated vesicles. Clathrin-deficient cells were also missing large translucent vacuoles that serve as endosomes and contractile vacuoles. In the absence of clathrin heavy chain, mutant cells displayed three distinct functional defects: (*a*) impairment in endocytosis of fluid phase markers, but competence in another endocytic pathway, the phagocytosis of solid particles; (*b*) defects in osmoregulation; and (*c*) inability to complete the starvation-induced development cycle.

CLATHRIN originally gained recognition as the basic subunit of polygonal lattices (Pearse, 1975, 1976) that control receptor-mediated endocytosis (Anderson et al., 1976). The past 16 years of research have focused on understanding the function of clathrin and clathrin-associated proteins in endocytosis (Keen, 1990). These studies indicate that the clathrin lattice has two main functions (Anderson, 1991; Goldstein et al., 1985); it converts planar segments of membrane into vesicles, and it clusters membrane receptors that use coated pits as vehicles for delivering substances to certain vesicular compartments. Understanding the molecular mechanisms that underlie these functions is a major emphasis of current day research (Brodsky, 1988; Keen, 1990; Pearse, 1988).

Receptor-mediated endocytosis is a phrase coined to describe the process of LDL binding, uptake, and degradation in human fibroblasts (Goldstein and Brown, 1974). We now know that cells use clathrin-coated vesicles in this pathway to internalize a variety of nutritionally important as well as opportunistic molecules from the plasma membrane (Anderson, 1991; Brodsky, 1988; Goldstein et al., 1985). In addition to endocytosis, cells use clathrin-coated vesicles to selectively transport specific proteins to secretory vesicles and lysosomes from the *trans*-golgi network (Brown and Farquhar, 1984; Orci et al., 1984; Tooze and Tooze, 1986). Much less understood is the use of coated pits to modulate hormone-generated signals (Schlessinger, 1980) or the function of clathrin at sites of phagocytosis (Aggeler and Werb, 1982).

An important experimental model that can give new insight into the function of coated membranes is a clathrin-deficient cell. To obtain such a cell, Doxsey et al. (1987) depleted the cytoplasm of CV-1 cells of functional clathrin by microinjecting antibodies against the clathrin heavy chain. The microinjected cells exhibited a 50% reduction in both bulk phase and receptor-mediated endocytosis without affecting the export of newly synthesized influenza hemagglutinin to the plasma membrane. While these experiments demonstrate at least a partial role for clathrin in endocytosis but not in constitutive secretion, conclusions are limited by the experimental constraints of microinjection experiments. For example, the degree of clathrin depletion cannot be measured. Moreover, because these clathrin-deficient cells cannot be propagated, the long-term effects of clathrin depletion on cell morphology and behavior cannot be studied.

Clathrin-deficient cells have also been created in the yeast *Saccharomyces cerevisiae*. The eviction of the clathrin heavy chain gene from this yeast generates cells that are either very slow-growing (Payne and Schekman, 1985) or dead (Lemmon and Jones 1987); depending on the particular yeast strain and its genetic background. Clathrin-minus yeast have been informative as to the role of clathrin in cellular processes such as the bulk constitutive secretion of plasma membrane proteins (which appears to be normal in clathrin-minus cells) and the localization of the Golgi-associated protease, Kex2p (which is not normal in clathrin-minus yeast) (Payne and Schekman, 1985, 1989). However, the relevance of many of the proposed functions for clathrin-coated vesi-

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cles cannot be tested in a yeast background. For example, studies of endocytosis and membrane recycling in yeast are hindered by the presence of a cell wall and low rates of endocytosis. Structural studies are similarly limited: coated pits and vesicles are not detected in electron micrographs of yeast.

We have accordingly focused our effort on developing an alternate experimental model in which to test clathrin function. Coated pits and coated vesicles are readily detected within the cytoplasm of *Dictyostelium discoideum*. (Swanson et al., 1981). In addition to these structural hallmarks of clathrin-mediated traffic, this eukaryotic microorganism exhibits many behavioral characteristics that are more typically found in complex eukaryotic cells including pinocytosis, phagocytosis, and a simple developmental cycle that is initiated by extracellular signals generated in response to starvation (Vogel, 1987; Loomis, 1982). In the current report, we have introduced antisense constructs in *Dictyostelium* to selectively inhibit clathrin heavy chain expression. Against this genetic and functional background, we found that clathrin-deficient *Dictyostelium* have multiple, informative defects.

Materials and Methods

Construction of the Vectors

The 2.8-kb BamHI-BglII fragment of the *Dictyostelium* clathrin heavy chain cDNA (O'Halloran and Anderson, 1992) was subcloned in both orientations behind the *Dictyostelium* actin 6 promoter of the neomycin-selectable plasmid pDNeoII (Witke et al., 1989), creating plasmids pTO103S and pTO103A. The control plasmid, pTO103S, generates a 3.0-kb RNA in the same orientation as wild-type clathrin heavy chain mRNA (sense RNA). Protein is not synthesized from this RNA due to the presence of an in frame stop codon between the promoter and the BamHI-BglII fragment. Plasmid pTO103A, which has the 2.8 BamHI-BglII fragment in the orientation opposite to pTO103S, generates RNA that is complementary to wild-type clathrin heavy chain mRNA (antisense RNA). The two plasmids were introduced into *Dictyostelium* cells (strain AX2) by calcium-phosphate precipitation followed by a glycerol shock (O'Halloran and Spudich, 1990), allowed to recover overnight in HL-5 (Sussman, 1987), and then cloned and propagated in HL-5 with 10 μg/ml of G418. Clonal colonies transformed with either plasmid pTO103S or pTO103A were grown thereafter in individual petri dishes in HL-5 that contained 10 μg/ml of G418.

Western Blots

Lysates of cells transformed with either a control plasmid, pTO103S, or with pTO103A were prepared and boiled with sample buffer as described previously (O'Halloran and Spudich, 1990). Protein (50 μg) from each sample was separated by electrophoresis on a 7% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunostained with a polyclonal anti-*Dictyostelium* clathrin heavy chain serum diluted 1/1,000 with buffer A (5% dried milk, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) followed by ^{125}I-labeled goat anti-rabbit IgG.

Northern Blots

RNA was prepared from cells according to Nellen et al. (1989) and 10-μg samples were separated by electrophoresis in the presence of formaldehyde on an agarose gel (Sambrook et al., 1989). The gel was transferred to a nylon membrane (ICN Biomedical, Irvine, CA) in 20 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) and then hybridized with a strand-specific probe labeled with alpha-^{32}PdATP. Single-stranded probes were synthesized from m13 templates that carried an 800-bp fragment of the clathrin heavy chain cDNA oriented in either direction (Sambrook et al., 1989).

Electron Microscopy

Samples of each cell type were prepared for thin section EM by the method of Swanson et al. (1981) except that samples were stained with 4% uranyl acetate and 2% lead citrate.

Measurement of Pinocytosis and Phagocytosis

To measure pinocytosis, cells were incubated with 2.5 μCi/ml ^{3}H-dextran (70,000 kD; specific activity, 542 mCi/g; Amersham Corp., Arlington Heights, IL) in HL-5 at 20°C. At various times, samples of 9×10^6 cells were diluted quickly into 10 ml of 0°C HL-5, washed three times with cold HL-5, lysed in Tris buffer (50 mM Tris, 20 mM sodium pyrophosphate, 5 mM EGTA, 5 mM EDTA, 1 mM PMSF, 0.5% Triton X-100, pH 7.5) and counted in a scintillation counter. Alternatively, cells were incubated in HL-5 that contained 2 mg/ml fluorescein-dextran (M_r 70,000; Molecular Probes, Eugene OR) for 1 h at 20°C, washed with HL-5, fixed with 3.7% formaldehyde in buffer B (17 mM Na_2HPO_4, 50 mM NaCl, pH 6.8) and then viewed with either phase or fluorescence optics. To measure phagocytosis, cells were incubated in HL-5 for 10 min at 20°C with bacteria that were labeled with tetramethylrhodamine (Molecular Probes, Eugene, OR), washed in HL-5, fixed with 3.7% formaldehyde in buffer B, and viewed with either phase or fluorescence optics. Photographs were taken with a Zeiss photomicroscope III using an automatic photometer. Exposure times for *chc*$^-$ cells and control cells were equivalent.

Development

Control or clathrin-deficient cells were grown axenically in HL-5 and allowed to attach to the surface of plastic dishes. The axenic media was replaced with starvation buffer (20 mM 2(N-morpholino)ethanesulfonic acid, 0.2 mM $CaCl_2$, 2 mM $MgSO_4$, 50 mM NaCl, pH 6.8) and incubated for the indicated time at 20°C. The cells were then viewed and photographed under phase optics.

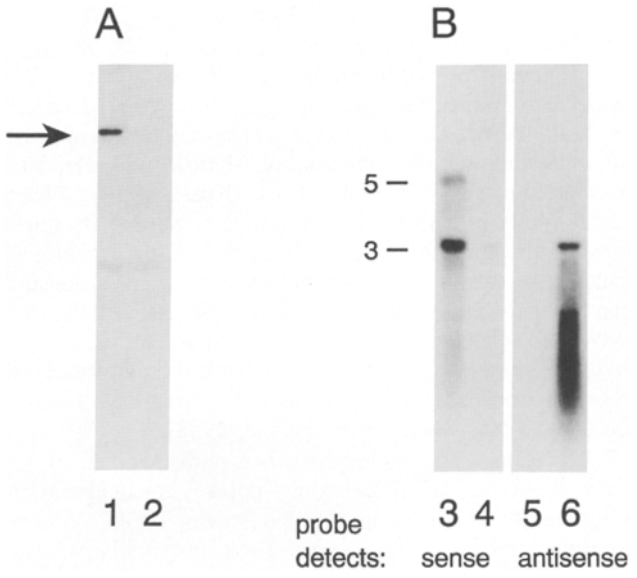

Figure 1. (*A*) Western blot analysis: 50 μg of protein from cell lysates transformed with either a control plasmid, pTO103S (lane *1*), or with the antisense plasmid pTO103A (lane *2*) were separated by electrophoresis on 7% SDS-polyacrylamide gels, transferred to nitrocellulose, and immunostained with a polyclonal anti-*Dictyostelium* clathrin heavy chain serum. The arrow indicates the position of the 190-kD clathrin heavy chain band. (*B*) Northern blot analysis: Lanes 3 and 5 contain RNA from cells transformed with control plasmid pTO103S whereas lanes 4 and 6 contain RNA from clathrin-deficient cells transformed with plasmid pTO103A. Lanes 3 and 4 were hybridized with a probe specific for sense clathrin RNA (*sense*), whereas Lanes 5 and 6 were hybridized with a probe specific for antisense clathrin RNA (*antisense*). The sizes of molecular markers for RNA are given in kilobases to the left of the gel.

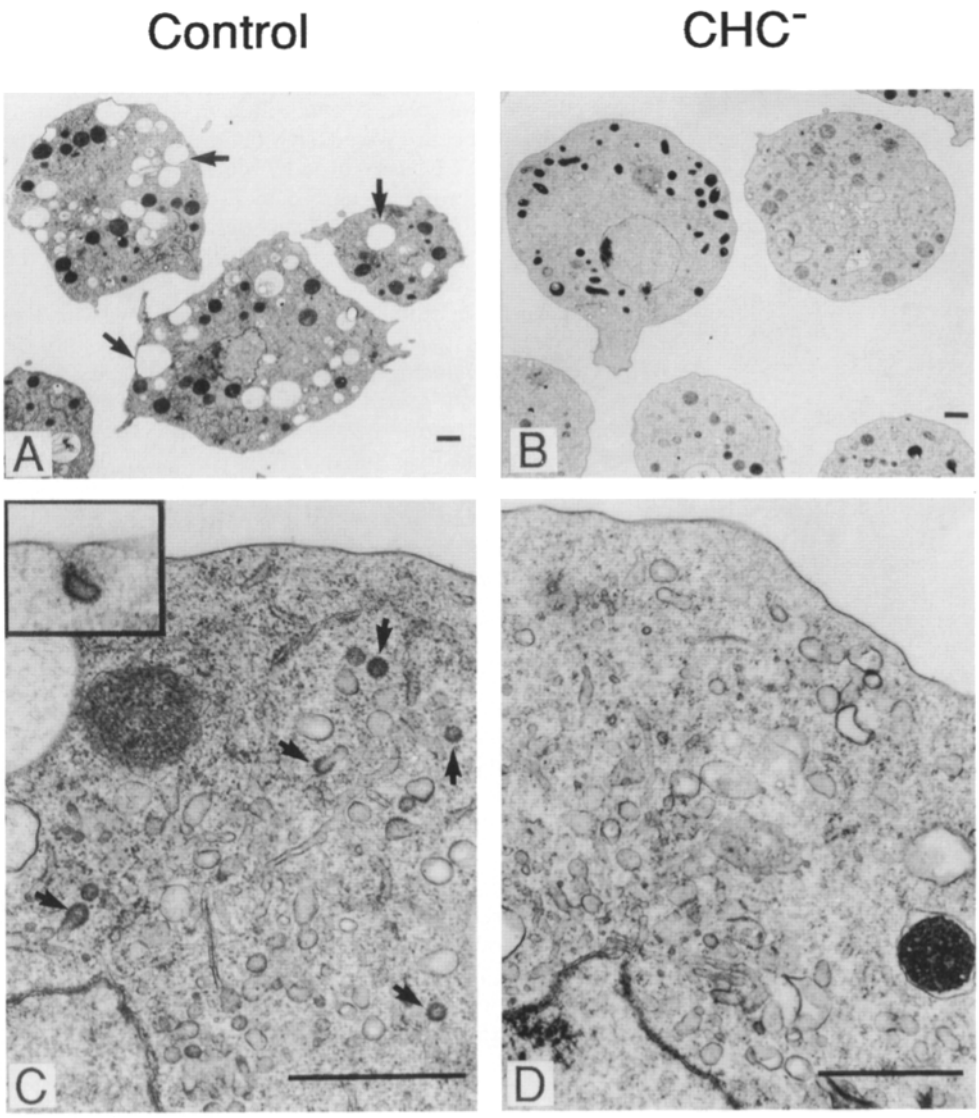

Figure 2. Ultrastructural appearance of control and clathrin-deficient *Dictyostelium*. Thin section micrographs of control (*A* and *C*) and clathrin-deficient cells (*B* and *D*) are shown. Large vacuoles present in control cells are indicated with arrows in *A*; these structures are absent in clathrin-deficient cells shown in *B*. Coated vesicles (*C*, *arrows*) and coated pits (*C*, *inset*) were identified in the control cells; these structures were missing in clathrin-deficient cells. Bars, 1 μm.

Results

Creation of Clathrin-deficient Cells

Clathrin-deficient cells were generated by transfecting *Dictyostelium discoideum* cells with a plasmid that would produce RNA complementary to the endogenous message encoded by the *Dictyostelium* clathrin heavy chain gene. The hybridization of the endogenous "sense" message with the transfected "antisense" strand should inhibit the synthesis of clathrin. The 2.8-kb BamHI–BglII fragment of the *Dictyostelium* clathrin heavy chain cDNA (O'Halloran and Anderson, 1992) was subcloned behind the *Dictyostelium* actin 6 promoter of the neomycin-selectable plasmid pDNeoII (Witke et al., 1989) in an "antisense" orientation to create plasmid pTO103A. As a control for the effects of transfection, a second plasmid, pTO103S, was constructed with the same BamHI–BglII fragment in the opposite, "sense" orientation behind the actin 6 promoter. The presence of an initiation ATG and stop codons preceded the clathrin heavy chain cDNA to ensure that clathrin heavy chain protein was not produced by either plasmid.

Dictyostelium strain AX2 was transfected with either plasmid pTO103A or pTO103S and grown under selection conditions. G418 resistant colonies were subcloned and maintained as individual colonies derived from a single cell. Initially, we noted that some of the colonies transfected with the antisense plasmid grew at about one-half the rate of wild type cells. When sufficient quantities of both the sense and the slow-growing antisense cells were available, we used a *Dictyostelium* specific anticlathrin heavy chain serum (O'Halloran and Anderson, 1992) in an immunoblot assay to analyze the cells for the expression of clathrin heavy chain. Fig. 1 *A* shows that cells expressing the sense strand (control cells transformed with pTO103S) had a single band of 190 kD (Fig. 1 *A*, lane 1). By contrast, cells that received the antisense plasmid pTO103A were devoid of any band at this molecular weight (Fig. 1 *A*, lane 2). Both sets of cells contained a 70-kD, nonspecific band, which served to ensure that equal amounts of protein were loaded in each lane. We estimate from the sensitivity of the immunoblot with this antibody that clathrin negative cells contained <3% of the wild type amounts of clathrin.

We next assayed clathrin positive cells (designated, control) and clathrin negative cells (designated, *chc*⁻) for the expression of both sense and antisense RNA (Fig. 1 *B*). Con-

trol cells contained both the endogenous 5-kb clathrin mRNA and the 3-kb RNA synthesized by the sense plasmid (Fig. 1 *B*, lane *3*); however, antisense RNA was not detected in these cells (Fig. 1 *B*, lane *5*). By contrast, the *chc*⁻ cells completely lacked clathrin heavy chain sense mRNA (Fig. 1 *B*). Instead they contained abundant antisense RNA (Fig. 1 *B*, lane *6*), as well as degradation products derived from this RNA.

Morphological Phenotype of chc⁻ Cells

Cells that lacked clathrin in the immunoblot assay were selected for further study. The control "sense" cells were indistinguishable from untransfected, wild-type cells. Compared to control cells, the *chc*⁻ cells appeared in the light microscope to have a more rounded shape. In addition, the size of the *chc*⁻ cells was much more variable. Many were slightly smaller than the control cells, and occasionally large multinucleated cells were observed.

Low power electron microscopic images showed that the two sets of cells differed in overall shape, but some organelles such as nuclei and mitochondria were identical (compare Fig. 2 *A* with *B*). A remarkable feature of wild-type, axenic strains of *Dictyostelium* is that they contain numerous large vacuoles (Fig. 2 *A*, *arrows*). Some of these are contractile vacuoles (de Chastellier et al., 1978; Patterson, 1980; Quiviger et al., 1978) that are thought to function in osmoregulation. Others are large endosomes, a prelysosomal compartment that receives membrane and internalized material during pinocytosis and phagocytosis (Padh et al., 1989). Comparison of 215 cross-sections of control cells with 312 cross-sections of *chc*⁻ cells revealed that wild-type cells were filled with large translucent vacuoles, whereas *chc*⁻ cells were almost completely devoid of these organelles (compare Fig. 2 *A* with *B*). Occasionally a few translucent vacuoles were detected in some *chc*⁻ cells (6% of all samples examined), although these tended to be smaller than those found in wild-type cells.

To determine whether *chc*⁻ cells contained coated vesicles and pits, we selected and enlarged vesicle-rich areas from individual cells. We counted 69 coated vesicles in the cytoplasm (Fig. 2 *C*, *arrows*) and 9 coated pits (Fig. 2 *C*, *inset*) along the plasma membrane in 37 areas selected from wild-type cells. These coated structures were not uniformly distributed but were often clustered near the nucleus or the plasma membrane. Coated vesicles were particularly enriched near the microtubule organizing center. We found an average of five coated vesicles associated with the six microtubule organizing centers we examined in wild-type cells. In contrast, coated vesicles were absent from *chc*⁻ cells (Fig. 2 *D*). No coated vesicles or pits were observed in 52 vesicle-rich areas enlarged from *chc*⁻ cells. The seven microtubule organizing centers we examined in *chc*⁻ cells were also completely devoid of coated vesicles. Other than the absence of large translucent vacuoles, coated pits and coated vesicles, all other aspects of *Dictyostelium* morphology appeared normal in *chc*⁻ cells.

Functional Defects in chc⁻ Cells

Dictyostelium cells can accomplish both phagocytosis, the internalization of solid particles, and pinocytosis, the internalization of fluid (Vogel, 1987). To test the contribution of clathrin heavy chain to these endocytic functions, we measured these processes in the mutant cells. We first assayed mutant and normal cells for their ability to phagocytose particulate material (Fig. 3, *A–D*). Both types of cells were incubated with rhodamine-labeled bacteria for 10 min at 20°C and viewed with either phase (Fig. 3, *A* and *C*) or fluorescence (Fig. 3, *B* and *D*) optics. Both sets of cells appeared to ingest the same number of bacteria (compare Fig. 3 *B* with *D*). Adjustment of the focal plane of the microscope

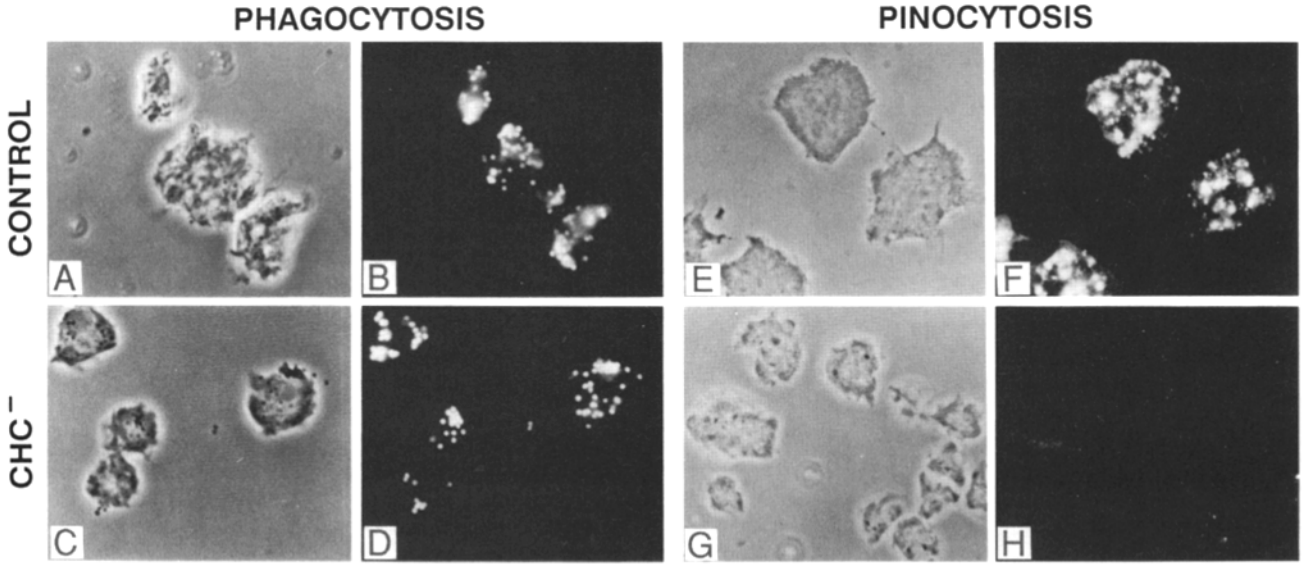

Figure 3. Endocytosis in control and clathrin-deficient cells. To measure phagocytosis (*A–D*), cells were incubated with rhodamine-labeled bacteria, washed, fixed, and viewed with either phase optics (*A* and *C*) or fluorescence optics (*B* and *D*). Both types of cells appeared equally proficient at internalization of bacteria. To measure pinocytosis (*E–H*), cells were incubated in media that contained 2 mg/ml fluorescein-dextran (M_r 70,000) for 1 h at 20°C, washed, fixed, and then viewed with either phase optics (*E* and *G*) or fluorescence optics (*F* and *H*). While control cells internalized and sequestered the fluid phase marker (*F*), clathrin-deficient cells failed to internalize the marker (*H*).

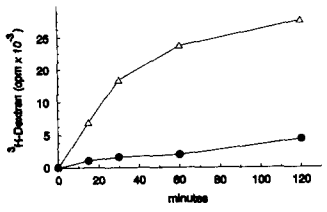

Figure 4. Pinocytosis of ^{3}H-dextran in control and clathrin-deficient cells. Control cells (*open triangles*) or *chc*$^-$ cells (*closed circles*) were incubated with 2.5 μc/ml ^{3}H-dextran media at 20°C. At various times, samples of 9 × 10^6 cells were diluted quickly into ice-cold buffer, washed, lysed, and counted in a scintillation counter. Clathrin-deficient cells were significantly impaired in pinocytosis.

confirmed that the bacteria had actually entered the *chc*$^-$ cells and not simply bound to the cell surface. We obtained similar results when cells were allowed to internalize rhodamine-labeled yeast (data not shown).

To examine pinocytosis, we next examined the ability of mutant and wild-type cells to internalize bulk phase markers by pinocytosis (Kayman and Clarke, 1983). Each set of cells was incubated with 2 mg/ml of fluorescent dextran for 1 h at 20°C before viewing with either fluorescence (Fig. 3, *F* and *H*) or phase optics (Fig. 3, *E* and *G*). Control cells avidly accumulated the fluorescent probe (Fig. 3 *E*). By contrast, there was an almost complete lack of dye uptake by the *chc*$^-$ cells (Fig. 3 *H*). After a thorough search, we found a few positive cells, but the intensity of fluorescence in these cells was far less than wild type cells.

To quantify the defect in pinocytosis in the mutant and wild type cells we then incubated the cells with ^{3}H-dextran for various times at 20°C (Fig. 4). Control cells steadily accumulated the ^{3}H-dextran for the first hour of incubation before net uptake plateaued (Fig. 4, *open triangles*). *Chc*$^-$ cells, on the other hand, internalized very little dextran during this period (Fig. 4, *closed circles*). Initial rates of internalization were eight times higher for control cells than for *chc*$^-$ cells.

Since some of the electron lucent vacuoles that were absent from *chc*$^-$ cells have been identified as contractile vacuoles that function in osmoregulation (de Chastellier et al., 1978), we compared the two cell types for their ability to adjust to osmotic stress. Both the *chc*$^-$ and the control cells were shifted from HL-5 media to a low ionic strength buffer (20 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.8). *Chc*$^-$ cells swelled and became round as they became engorged with water (Fig. 5, compare *top left* and *middle*). Subsequently, *chc*$^-$ cells appeared to adjust to their osmotic environment and returned to their original size (Fig. 5, *top right*). Under identical conditions, control cells were able to compensate for the sudden change in their osmotic environment and regained their original size and shape (Fig. 5, *bottom*).

When placed in an environment devoid of nutrients, wild-type *Dictyostelium* initiate a simple developmental program. Using pulses of extracellular cAMP as chemotactic cues, dispersed cells migrate together to form an aggregation center. This mass of cells ultimately transforms into a fruiting body consisting of two basic cell types: stalk cells and spore cells. Since clathrin-coated pits have been implicated in modulating hormonal signals (Schlessinger, 1980), we were interested in knowing whether *chc*$^-$ cells would undergo this developmental pattern. Both *chc*$^-$ and control cells were washed free of nutrients and incubated with starvation buffer. Cells were inspected at various times with a light microscope (Fig. 6). Wild-type cells initially became elongated and formed streams of migrating cells (Fig. 6; control, 5 h). After 18 h, these cells had formed a well-developed aggregation center (Fig. 6; control, 18 h). By contrast, *chc*$^-$ cells

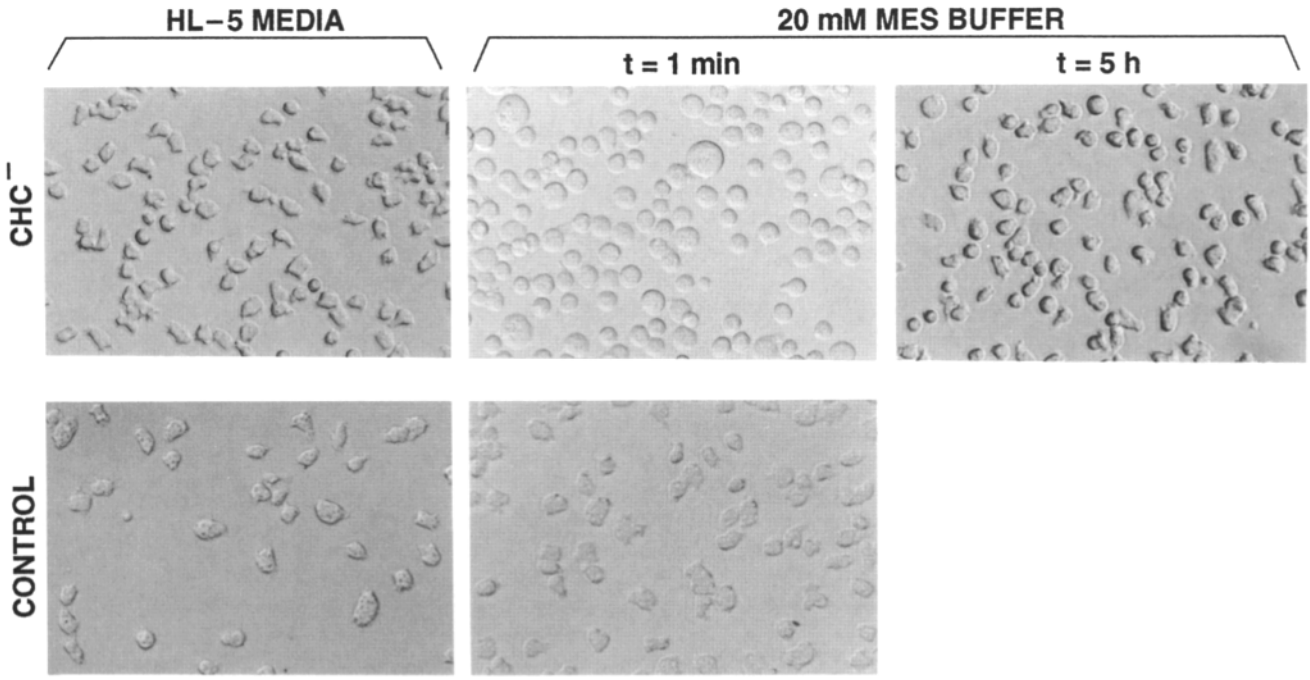

Figure 5. Response of clathrin-deficient and control cells to low osmotic buffer. Clathrin-deficient (*top, CHC*$^-$) or control (*bottom, CONTROL*) cells growing in HL-5 media (*left*) were shifted to 20 mM Mes buffer, pH 6.5, and photographed at the indicated times under phase optics. The 1 min timepoint (*middle*) shows that clathrin-deficient cells quickly swelled in this low ionic strength environment whereas the size and shape of control cells was unaffected. By 5 h in 20 mM Mes buffer, clathrin-deficient cells had resumed their original size and shape (*top right*).

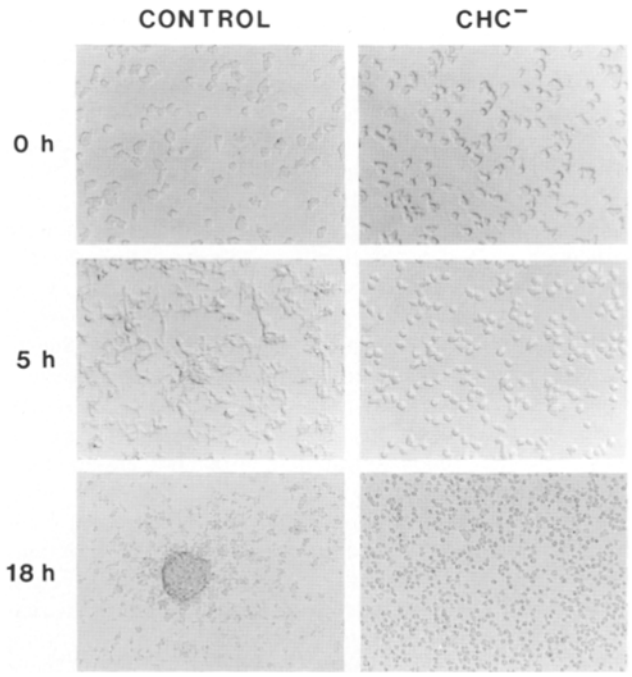

Figure 6. Development response of control and clathrin-deficient cells. Control (*CONTROL*) or clathrin-deficient (*CHC⁻*) cells were allowed to attach to the surface of plastic dishes. The nutritive media was replaced with starvation buffer to induce development and cells were photographed at the times indicated on the left. While control cells chemotactically streamed together (5 h) to form a mound (18 h), clathrin-deficient cells failed to respond to starvation and did not form streams or mounds.

appeared to be completely blocked in development (Fig. 6, *chc⁻*). They failed to undergo even the earliest stages of development, including the initial shape change (Fig. 6, *chc⁻* 0 h, 5 h) and directed cell migration (Fig. 6, *chc⁻* 5 h, 18 h).

Discussion

Clathrin-deficient *Dictyostelium* cells are viable. However clathrin-deficient *Dictyostelium* cells display at least three distinct functional defects: (*a*) inability to take up bulk phase markers by pinocytosis; (*b*) impaired osmoregulation; and (*c*) inability to enter a developmental cycle. These properties do not appear to be strain-specific: clathrin-deficient cells from both the AX2 and the AX3 strains of axenic cells had identical phenotypes (data not shown). Further, these characteristics were directly correlated with the lack of clathrin heavy chain protein. When *chc⁻* revertants were selected by growing the cells without G418, the wild-type phenotype returned concurrently with the expression of clathrin heavy chain (data not shown).

This study provides genetic evidence that the clathrin heavy chain is important for fluid phase pinocytosis. Several studies have shown that an alternate, actin-dependent uptake pathway functions in certain cells (Hansen et al., 1991; Sandvig and van Deurs, 1991; van Deurs et al., 1989). If a route for actin-mediated pinocytosis exists in *Dictyostelium*, it apparently cannot operate independently of the clathrin-mediated route. Actin-based pathways of internalization are not inactivated in the *chc⁻* cells. Mutant cells remained able to internalize particulate material, a process that in *Dictyostelium* is dependent on both actin and myosin I (Fukui et al., 1989; Hammer and Jung, 1990). Therefore, the most direct explanation for the contribution of clathrin to pinocytosis is that clathrin-coated pits carry into cells fluid that is fortuitously trapped during coated vesicle formation at the cell surface.

The large translucent vacuoles that are missing in *chc⁻* cells include both endosomes that receive internalized material (de Chastellier and Ryter, 1983; Padh et al., 1989) and contractile vacuoles that regulate osmotic pressure (de Chastellier et al., 1978; Patterson, 1980; Quiviger et al., 1978). The disruption of membrane traffic due to the absence of coated vesicle formation most likely accounts for the deficiency in these organelles. This implies that there is a constant flux of membrane through these vacuoles and that clathrin heavy chain is required for the import of this membrane to endosomes and contractile vacuoles. The formation of endosomes from clathrin-coated vesicles has been shown in many other systems (Goldstein et al., 1985). However, why clathrin-coated vesicles are required for the presence of contractile vacuoles is less clear. Morphological studies of algal cells have noted coated vesicles in proximity to contractile vacuoles, suggesting that these vesicles might mediate membrane flow to maintain the contractile vacuole (Manton, 1964; Weiss, 1983). In *Dictyostelium* cells, clathrin-coated vesicles may retrieve contractile vacuole membrane from the plasma membrane after the vacuole has fused to expel water. Alternatively, clathrin-coated vesicles could play an essential role in the biogenesis of contractile vacuoles; for example clathrin-coated vesicles from the *trans*-golgi network may be required to transport proteins or membrane that are essential for contractile vacuole formation.

Dictyostelium discoideum are found naturally in the low osmotic environment of moist forest soil. Without a rigid cell wall, this organism must constantly excrete water to maintain cell volume. This is accomplished by water retrieving contractile vacuoles (Patterson, 1980). The current study confirms that these vacuoles play a role in water excretion because *chc⁻* cells, which appear to lack this organelle, were unable to normally regulate their cell volume when placed in a low ionic strength medium. Endocytic compartments in more complex organisms also appear to be involved in water transport. Clathrin-coated vesicles and endosomes in kidney collecting duct cells house water transporters that are recruited to the cell surface in response to antidiuretic hormone (Brown and Orci, 1983; Brown et al., 1988; Verkman et al., 1989). Thus, the involvement of clathrin-coated vesicles in water transport appears to be common to simple unicellular organisms as well as highly specialized cells of mammalian kidney.

Except for the morphological differences that were described, *chc⁻* cells look relatively normal. We were surprised, therefore, that *chc⁻* cells were completely unable to enter into the developmental program. One possible explanation for this behavior is that essential signaling molecules such as cAMP receptors (Klein et al., 1988) were not present on the surface of *chc⁻* cells due to impaired transport of membrane proteins from the Golgi. A more intriguing possibility is that coated pits play an essential role in signal transduction. Modulation of hormonal signals can be achieved in part by down regulating receptors through receptor-mediated endocytosis (Schlessinger, 1980). In fact, the cAMP receptor in *Dictyostelium* can be down regulated by inter-

nalization in response to the stimulation of developing cells with cAMP (Wang et al., 1988). Regulation of receptor number, as well as the spatial arrangement of the receptors on the cell membrane (Bretscher, 1984), may be a critical architectural feature of the cell surface that allows the cell to move in the direction of a chemotactic gradient. A final possibility is that coated pits are directly required for cell migration. Coated pits have been found in association with cell–substratum attachment sites (Nermut and Burt, 1991; Nermut et al., 1991), although their exact function at this location is not known. Conceivably, they play a role in modulating the activity of adhesive molecules that are essential for cell movement (Bretscher, 1989; Sczekan and Juliano, 1990). If so, this function would be impaired in the *chc*$^-$ cells. Clearly the dramatic phenotype of the *chc*$^-$ cells offers a unique opportunity to better understand the role of endocytosis in development.

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