Clathrin heavy chain is required for spore cell but not stalk cell differentiation in *Dictyostelium discoideum*

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SUMMARY

Previous studies of a clathrin-minus *Dictyostelium* cell line revealed important roles for clathrin heavy chain (clathrin) in endocytosis, secretion of lysosomal hydrolases and osmoregulation. In this paper, we examine the contribution of clathrin-mediated membrane traffic to development in *Dictyostelium discoideum*. Clathrin-minus cells were delayed in early development. When exposed to starvation conditions, clathrin-minus cells streamed and aggregated more slowly than wild-type cells. Although clathrin-minus cells displayed only 40% the level of extracellular cyclic AMP that bind specifically to cyclic AMP receptors from the plasma membrane may be one mechanism for regulating and coordinating chemotaxis and cell movement during early development (Wang et al., 1988). Endocytosis of other membrane proteins may be of general importance for *Dictyostelium* development since the profile of glyco-­proteins on the plasma membrane of *Dictyostelium* cells changes dramatically throughout the 24 hour developmental cycle (Burridge and Jordan, 1979). The regulated secretion of spore coat proteins from the prespore vesicle, a specialized vesicle in prespore cells that contains many of the necessary constituents of the spore coat, is a late developmental event that requires directed membrane traffic (West and Erdos, 1992). All of these studies suggest that membrane traffic is important in *Dictyostelium* development, however, to date, the contribution of genes involved in selective membrane traffic have received little attention in *Dictyostelium* or other developmental systems.

One of the more fully characterized mechanisms of selective membrane traffic is the directed movement of proteins and membranes by vesicles coated with clathrin. We recently cloned the single *Dictyostelium* clathrin heavy chain (*chcA*) gene (O’Halloran and Anderson, 1992b), and used anti-sense (O’Halloran and Anderson, 1992a) and gene disruption (Ruscetti et al., 1994) techniques to generate mutant cells devoid of clathrin heavy chain protein. Studies of the clathrin-minus cells during growth identified roles for clathrin in endocytosis, secretion of lysosomal hydrolases and osmoregulation. In this paper, we examine the contribution of clathrin-mediated membrane traffic to development in *Dictyostelium discoideum*. Clathrin-minus cells were delayed in early development. When exposed to starvation conditions, clathrin-minus cells streamed and aggregated more slowly than wild-type cells. Although clathrin-minus cells displayed only 40% the level of extracellular cyclic AMP receptors normally found in wild-type cells, they responded chemotactically to extracellular cyclic AMP. Clathrin-minus cells down-regulated cyclic AMP receptors, but only to half the extent of wild-type cells.

We found that the extent of development of clathrin-minus cells was variable and influenced by environmental conditions. Although the mutant cells always progressed beyond the tipped mound stage, the final structure varied from a finger-like projection to a short, irregular fruiting body.
osmoregulation and the regulated secretion of the lysosomal hydrolase, α-mannosidase (O’Halloran and Anderson, 1992b; Ruscetti et al., 1994). Here, we used clathrin-minus cells to explore the role of clathrin in development. We found that clathrin-minus cells assembled into mounds more slowly and were compromised in their ability to construct a mature fruiting body. Clathrin-minus cells inefficiently sorted prestalk from prespore cells. Despite this defect, the mutants were able to complete differentiation of stalk cells, but displayed a complete block in their ability to differentiate into mature spore cells.

**MATERIALS AND METHODS**

**Growth and creation of cell lines**

*Dictyostelium* strain Ax2 and mutant and control cell lines were cultured on Petri dishes in HL5 media (0.75% proteose peptone (Difco Laboratories, Inc., Detroit, MI), 0.75% BBL thiotone E peptone (Becton Dickinson, Cockeysville, MD), 0.5% Oxoid yeast extract (Unipath, LTD., Basingstoke, Hampshire, England), 1% glucose, 2.5 mM NaH2PO4, and 8.8 mM KH2PO4, pH 6.5) supplemented with penicillin-streptomycin (GIBCO BRL, Gaithersburg, MD) at 60 U/ml. *Dictyostelium* clathrin heavy chain-minus cell line HO101 and wild-type cell line HO102 have been described previously (Ruscetti et al., 1994). Three new clathrin-minus cell lines, HO103, HO105 and HO106, and a wild-type control cell line HO104 were created from strain Ax2 in a similar manner by substituting the 1.4 kb *Pst*-NcoI fragment from the *Dictyostelium* clathrin heavy chain gene (O’Halloran and Anderson, 1992b) with the 1.4 kb *BamHI-BglII* fragment containing the blasticidin resistance gene (Sutoh, 1993). Transformants were generated and characterized as previously described (Ruscetti et al., 1994), except that the selection was 5 μg/ml blasticidin (ICN Biomedicals, Inc., Irvine, CA). Once the cell lines were determined to be stable, selective pressure was removed. Clathrin-minus or wild-type cell lines were transformed with promoter/lacZ reporter constructs using electroporation (Ruscetti et al., 1994) and selected for growth in HL5 containing 10 μg/ml G418 (GIBCO, BRL) and 60 U/ml penicillin and streptomycin (GIBCO BRL). Reporter constructs were *ecmA-lacZ*, *ecmB-lacZ* (Jermyn and Williams, 1991), *pxA-lacZ* (Dingermann et al., 1989) and *cotB-lacZ* (Fosnaugh and Loomis, 1993).

**Development and lacZ staining**

To examine development, cells were harvested and washed twice with the starvation buffer PDF (11 mM K2HPO4, 13.2 mM KH2PO4, 20 mM KCl, 1 mM CaCl2, and 2.5 mM MgSO4, pH 6.4). Cells were either plated on 1% Noble agar (Difco Laboratories, Inc.) plates made with PDF, or on a 0.45 μm HA Millipore filter over two absorbent pads (Millipore Corporation, Bedford, MA) (Sussman, 1987). Moist filters favored the construction of 'finger-like' projections while saturated filters favored development of structures that resembled fruiting bodies. Cells were stained for expression of promoter-*lacZ* reporter constructs as previously described (Dingermann et al., 1989). Structures from various stages of development were visualized under a coverslip using a Zeiss Axiophot microscope and Nomarski optics or directly using a Leica Wild M8 microscope and a Hamamatsu color saturated filters favored development of structures that resembled fruiting bodies. Cells were stained for expression of promoter-*lacZ* reporter constructs as previously described (Dingermann et al., 1989). Structures from various stages of development were visualized under a coverslip using a Zeiss Axiophot microscope and Nomarski optics or directly using a Leica Wild M8 microscope and a Hamamatsu color chilled 3CCD camera and controller (Hamamatsu, Bridgewater, NJ).

**cyclic AMP assays**

cyclic AMP-binding assays were performed as described previously (Van Haastert et al., 1992). Briefly, clathrin-minus or wild-type cells were washed and plated in PDF buffer. At various time points, cells were harvested, washed and resuspended in PDF. 10 mM DTT and 50,000 cts/minute [3H]cyclic AMP (Amersham, Life Sciences, Inc., Arlington Heights, IL) was added to each sample. Cells were mixed with cyclic AMP on ice for 30 seconds, then 1 ml of cold saturated ammonium sulfate was added to stabilize cyclic AMP binding. The

The cells were washed onto a glass filter, dried overnight, solubilized at 37°C with 5 ml Solvable (NEN Research Products, Boston, MA), and neutralized with 60 μl acetic acid. Scintillation fluid was added and the samples were counted in a scintillation counter. The binding activity of duplicate samples was averaged and the non-specific binding (in 200 μM cyclic AMP) was subtracted from specific binding (in 200 nM cyclic AMP) and plotted as the per cent maximal cyclic AMP binding. For down-regulation assays, wild-type and clathrin-minus cells were starved for 12 and 21 hours, respectively, to allow maximal expression of cyclic AMP-binding sites. Cells were harvested, counted and then shaken with or without 100 μM cyclic AMP. At hourly intervals, aliquots were measured as above for cyclic AMP binding. Data was expressed as the per cent of binding compared to the zero time point.

**Mixing experiments**

Clathrin-minus or control cells were mixed with wild-type cells at various ratios, plated on HA filters and allowed to develop for 48 hours. Plates containing 10% clathrin-minus cells carrying a promoter/lacZ reporter construct plus 90% untransformed wild-type cells were fixed and stained for lacZ expression as described above. For analysis of spore formation, clathrin-minus and wild-type cells were mixed at various ratios and allowed to develop. Spores were harvested, treated with 0.5% Triton X-100 for 5 minutes, washed three times in PDF and plated in HL5 in 96-well tissue culture plates (Falcon, Becton-Dickinson). After incubating overnight, the culture was divided into two plates and HL5 containing 5 μg/ml blasticidin was added to one plate. Germination and growth was monitored for several weeks.
Northern analysis

Total RNA was prepared as described (Nellen et al., 1987) from clathrin-minus and wild-type cells at different times during development. 10 μg of total RNA was separated on a formaldehyde gel, transferred to Hybond N+ (Amersham), and probed with the following gene fragments: ecmA (2.4 kb EcoRI/HindIII fragment) (Williams et al., 1987), ecmB (2.4 kb EcoRI/HindIII fragment) (Ceccarelli et al., 1987), psA (1.3 kb HindIII/SphiI fragment) (Early and Williams, 1988), cotB (2.1 kb BamHI/HincII fragment) (Haberstroh and Firtel, 1990), spIA (0.3 kb EcoRI/XbaI fragment) (Richardson et al., 1991) and cIIA (casein kinase II α) (1.3 kb EcoRI/Xhol fragment) (Kikkawa et al., 1992). The blot was washed with 0.2x SSC and 0.1% SDS at 65°C, then exposed either to Kodak X-Omat film overnight or to phosphorimager plates for 2-4 hours followed by analysis with a Fujix BAS 1000 Bio Imaging Analyzer (Fujix, Japan).

RESULTS

Creation of new clathrin-minus cell lines

We previously created the clathrin heavy chain-minus (clathrin-minus) cell line, HO101, by ablation of the single chcA gene with the neomycin resistance gene (Ruscetti et al., 1994). For this work, we created three additional clathrin-minus cell lines (HO103, HO104 and HO105) using a blasticidin resistance gene as the selectable marker (Sutoh, 1993). We also selected a control cell line, HO106, which carries the blasticidin resistance gene, but expresses wild-type levels of clathrin heavy chain. Southern analysis of the clathrin-minus cell lines showed ablation of a functional clathrin heavy chain gene in these mutants while western and northern analysis demonstrated an absence of clathrin heavy chain protein and message (data not shown). Phenotypic analysis showed that development was similar in all clathrin-minus mutant cell lines.

Early development of clathrin-minus cells is delayed but normal

The first developmental event that occurs in response to starvation is the mass migration of about 100,000 cells into a multicellular mound. Clathrin-minus cells were able to initiate early development, but took longer to begin streaming in comparison to wild-type cells (Fig. 1). For example, after 7.5 hours of development, the distribution of clathrin-minus cells appeared uniform (Fig. 1A), whereas wild-type cells had gathered into streams of aggregating cells (Fig. 1B). With longer periods of time, clathrin-minus cells also streamed and aggregated together into a mound of cells (Fig. 1C-D). Close observation showed that clathrin-minus cells adopted the elongated and polarized morphology characteristic of wild-type streaming cells (data not shown). Relative to wild-type cells, however, more clathrin-minus cells lagged behind and failed to aggregate into a mound (Fig. 1E versus 1F).

To examine more closely the molecular events that occur in early development, we examined the binding of cyclic AMP to

Fig. 2. cAMP binding and down-regulation in clathrin-minus and wild-type cells. (A) cAMP-binding activity was measured in cells that had been starved for up to 12 or 24 hours. The % cAMP bound was normalized to the maximal amount of cAMP-binding activity measured for wild-type cells. Wild-type cells (triangles) reached their maximal cAMP-binding activity at 12 hours. Clathrin-minus cells (circles) reached their maximal cAMP-binding activity at 21 hours at levels 40% of wild-type binding. One representative experiment is shown. (B) Down-regulation of cAMP-binding sites was measured in clathrin-minus or wild-type cells that had been exposed to high amounts of cAMP displayed down-regulation of cAMP-binding activity within one hour. One representative experiment is shown.

Fig. 3. Development of clathrin-minus cells. (A) Fruiting bodies typically formed by wild-type cells. A large, round sorus was at the top of a long, thin stalk. (B) Finger-like structures formed by clathrin-minus cells plated on a filter moistened with PDF. In comparison with wild-type fruiting bodies, these structures were thick, irregular in shape, and lacked a distinct sorus or stalk. (C) Fruiting body-like structures (indicated with an arrow), formed by clathrin-minus cells plated on a filter saturated with PDF. Scale bar, 200 μm.
receptors on the surface of clathrin-minus or wild-type cells. We induced development in parallel cultures of clathrin-minus or wild-type cells and then measured the amount of \(^{3}H\)cyclic AMP bound to the surface of the cells. We found that the maximum level of cyclic AMP binding associated with clathrin-minus cells was expressed later (21 hours versus 12 hours) and at levels only 40% the level seen in wild-type cells (Fig. 2A).

One mechanism contributing to the down-regulation of cyclic AMP receptors has been hypothesized to be clathrin-mediated internalization (Wang et al., 1988). Therefore, we examined the ability of clathrin-minus cells to down-regulate cyclic AMP receptors. Clathrin-minus and wild-type cells expressing the maximum number of cyclic AMP-binding sites were exposed to high levels of cyclic AMP. The subsequent down-regulation of cyclic AMP-binding activity was measured. The time course of the disappearance of cyclic AMP-binding activity from the cell surface, shown in Fig. 2B, demonstrates that clathrin-minus cells do down-regulate cyclic AMP receptors, but only to half the extent of wild-type cells. Clathrin-minus and wild-type cells down-regulated cyclic AMP-binding activity to 60% and 30%, respectively, of the initial level of cyclic AMP binding within 1 hour. This effect was stable in both cell lines for over 4 hours and was not seen if cells were not exposed to continuous high levels of cyclic AMP. Thus while clathrin is not a primary mechanism for down-regulating cyclic AMP receptors, it may make the process more efficient.

Late development of clathrin-minus cells is impaired

During the development of wild-type Dictyostelium cells, a single tip emerged from the mound and elongated to form an extended finger. Under certain environmental conditions, the elongated finger became a pseudoplasmodium that migrated before completing development. Otherwise, the finger developed directly into a fruiting body consisting of a long slender stalk topped by a round sorus filled with spore cells (Fig. 3A). Wild-type development was completed within 24-28 hours.

While able to aggregate into mounds in early development, clathrin-minus cells were drastically compromised in their ability to progress beyond the mound stage. In contrast with wild-type cells, clathrin-minus cells were slower to develop, formed abnormal structures and were strongly influenced by environmental conditions. Development of the mutant cells was completed by 36-48 hours, which is about 12-24 hours longer than wild-type cells. On bacterial lawns, PDF-agar plates and moist PDF filters, clathrin-minus cells terminated development with the formation of long “finger-like” projections (Fig. 3B) that lacked a distinct sorus or stalk. Unlike wild-type fingers, the clathrin-minus projections never formed migrating pseudoplasmodia. On filters saturated with PDF buffer, clathrin-minus mutants often formed structures that had a rounded sorus on top of an elongated projection (arrow, Fig. 3C). In contrast with wild-type fruiting bodies (Fig. 3A), these structures were shorter with a smaller, irregularly shaped sorus and a thicker stalk.

Clathrin-minus cells form stalk cells but not spore cells

Wild-type fruiting bodies are built from two differentiated cell types, stalk cells and spore cells. We examined terminally developed structures with Nomarski and phase microscopy to look for the presence of these cells. The mature fruiting body formed by wild-type cells (Fig. 4D-F) had two morphologically distinct cell types. Stalk cells were highly vacuolated and

![Fig. 4. Differentiation of clathrin-minus stalk but not spore cells. Clathrin-minus or wild-type cells were plated under starvation conditions. Developed structures were placed under a coverslip and photographed under Nomarski (A,B,D,E) or phase (C,F) optics. (A) Clathrin-minus fruiting bodies structures showed amoeboid-appearing cells surrounding the stalk. In many cases, the stalk was completely obscured by the amoeboid cells. (B) Higher magnification of the structure shown in A, demonstrating the presence of vacuolated stalk cells and the absence of spore cells. (C) Phase photomicrograph of a clathrin-minus structure showing a row of vacuolated stalk cells inside a mass of amoeboid cells. (D) Mature wild-type fruiting bodies had a long thin stalk composed of vacuolated cells and a sorus containing ellipsoid spore cells. (E) Higher magnification of two fruiting bodies that were mechanically disrupted. Two stalks, positioned horizontally, contained vacuolated stalk cells. Spore cells, which were scattered from a burst sorus, were ellipsoid in shape and refractile. (F) Phase photomicrograph of a wild-type fruiting body with a cluster of ellipsoid spore cells atop a row of stalk cells. Scale bar, 10 \(\mu\)m.]

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arranged in rows along the stalk tube. Spore cells were smaller, ellipsoidal in shape and clustered together in a sorus that topped the stalk.

The clathrin-minus structures contained one terminally differentiated cell-type, the stalk cell, and a second population of cells that appeared undifferentiated. Embedded in the central core of finger-like structures or in the stalk of fruiting body-like structures were differentiated cells with a morphology characteristic of mature stalk cells (Fig. 4A-C). These clathrin-minus stalk cells were arranged in rows and were highly vacuolated. The cells surrounding the central core of stalk-like cells resembled undifferentiated amoebae and were amorphous in appearance, not oval and phase-lucent like wild-type spore cells (compare Fig. 4B with E, and Fig. 4C with F). Furthermore, these amorphous cells lysed when treated with detergent, confirming that they were not mature spores that are resistant to detergent (data not shown). In summary, the cellular morphology suggested that clathrin-minus cells were capable of differentiating into stalk cells but not spore cells.

**Clathrin-minus cells express appropriate markers but fail to sort efficiently**

The inability of clathrin-minus cells to form spores could be due to a failure of the clathrin-minus cells to receive or respond to the proper signals for cell-type differentiation. If this were the case, expression of prespore genes might be altered or absent. To examine cell-type-specific gene expression, we performed northern analysis on clathrin-minus and wild-type RNA prepared from a developmental time course. We used probes specific for prestalk genes ecmA and ecmB, prespore genes psA and cotB, and sporulation gene spiA (Fig. 5). We found that prestalk-specific cell genes ecmA and ecmB were expressed in clathrin-minus cells at the same time (within 2 hours) and at levels comparable to wild-type levels of expression. The expression level of prespore genes psA and cotB were also comparable with wild-type levels although the onset was delayed slightly. The clathrin-minus cells, however, completely failed to express the sporulation gene spiA.

To examine the spatial fate of differentiated cells, we transformed mutant and wild-type cells with promoter/lacZ reporter constructs specific for prespore and prestalk expression. Wild-type cells displayed the expected patterns for both cell types, Cells expressing either the prestalk ecmA/lacZ or ecmB/lacZ reporter constructs migrated to the stalk and the lower and upper cups surrounding the sorus (Fig. 6C,F) (Jermyn and Williams, 1991). EcmB-expressing cells also localized to the basal disc (data not shown). Cells expressing the prespore psA construct localized exclusively in the sorus (Fig. 6I) (Dingermann et al., 1989).

In contrast with the defined cell-type patterns found in wild-type fruiting bodies, much less specific distributions of cell-types were found in the structures formed by clathrin-minus cells. When clathrin-minus mutants formed fruiting body-like structures, prestalk-expressing cells did localize correctly in the upper cup and stalk tube (Fig. 6B) as well as the basal disc below the stalk tube (Fig. 6E), but many prestalk ecmA- and ecmB-expressing cells were also scattered randomly throughout the structure. Indeed many of the finger-like terminal structures showed only a random distribution of prestalk-expressing cells (Fig. 6A,D). Sorting of prespore cells was similarly inefficient. In structures that resembled fruiting bodies, many prespore psA-expressing cells localized to a round sorus-like structure. Many psA-expressing cells also localized inappropriately throughout the stalk tube and the base of the terminal structures (Fig. 6G,H).

Wild-type cells can provide an environment that allows some Dictyostelium mutants to develop normally (Sussman, 1987). To test whether clathrin-minus cells would sort properly in a wild-type fruiting body, we examined the development of a mixture of cells containing 10% clathrin-minus cells and 90% wild-type cells. The mutant cells carried prestalk cell or prespore cell-type-specific lacZ reporter constructs. The small numbers of clathrin-minus cells in these mixtures did not perturb the formation of wild-type fruiting bodies.

The first marker that we examined was the prestalk ecmAllacZ reporter construct. Clathrin-minus ecmA-expressing cells showed normal patterning during the mound stage, but were inefficiently incorporated into fruiting bodies. More specifically, clathrin-minus ecmA-expressing cells were scattered randomly throughout the mound (Fig. 7A) as were wild-type cells (Fig. 7E). During finger formation, ecmA-expressing clathrin-minus cells remained concentrated at the base of the structure (Fig. 7B) while wild-type cells expressing ecmA localized to the tip of the culminating finger (Fig. 7F). In mature fruiting bodies, most ecmA-expressing clathrin-minus cells remained at the base of the fruiting body and lower stalk (Fig. 7D), with some cells in the lower part of the sorus (Fig. 7C). Wild-type ecmA-expressing cells were seen in the upper and lower cups and stalk, but rarely in the basal disc (Fig. 7G).

The second marker that we examined was the prestalk ecmBlacZ reporter construct. As with ecmA-expressing cells, both clathrin-minus and wild-type ecmB-expressing cells were randomly distributed throughout mounds (Fig. 8A,E), but
clathrin-minus cells lagged behind during culmination. During finger formation, clathrin-minus ecmB-expressing cells were localized to the lower third of the finger (Fig. 8B), whereas wild-type ecmB-expressing cells were concentrated in the posterior prestalk zone and scattered throughout the prespore zone (Fig. 8F). In mature fruiting bodies, some clathrin-minus ecmA-expressing cells were found in the sorus (Fig. 9C) but most remained in the basal disc (Fig. 9D). Wild-type ecmA-expressing cells were in the upper and lower cups and stalk (G). Scale bar, 50 μm.

A final set of experiments were done to determine the spatial fate of prespore psA-expressing cells mixed with wild-type cells. Clathrin-minus psA-expressing cells localized inefficiently to the sorus. Both clathrin-minus and wild-type psA-expressing cells were randomly distributed throughout early mounds (Fig. 9A,E). During culmination, clathrin-minus psA-expressing cells were localized only in the lower portion of the finger (Fig. 9B), instead of distributing throughout the entire prespore area (Fig. 9F). In mature fruiting bodies, some clathrin-minus psA-expressing cells were found in the sorus (Fig. 9C) but most remained in the basal disc (Fig. 9D). Wild-type cells expressing psA/lacZ localized entirely to the sorus as expected (Fig. 9G).

**Clathrin-minus cells are impaired in their ability to sporulate**

The psA/lacZ reporter experiments demonstrated that a small number of clathrin-minus prespore cells localized properly to

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**Fig. 6.** Expression of prespore and prestalk genes in clathrin-minus (A,B,D,E,G,H) and wild-type (C,F,I) cells. Cells expressing prestalk ecmA (A-C), prestalk ecmB (D-F) and prespore psA-lacZ (G-I) were developed for 24-48 hours and stained for lacZ expression. Clathrin-minus finger-like projections (A,D,G) showed scattered expression of all three developmental genes. In clathrin-minus structures carrying the psA/lacZ reporter construct (G), there also was a concentration of psA-expressing cells at the tip of each projection. Clathrin-minus fruiting bodies (B,E,H) showed some cell-type sorting, although many ecmA- (B), and ecmB- (E) and psA- (H) expressing cells were scattered or remained behind in the basal disc. The normal expression patterns of ecmA (C), ecmB (F), and psA (I) are shown in wild-type fruiting bodies for comparison. Scale bar, 50 μm.

**Fig. 7.** Localization of clathrin-minus prestalk ecmA-expressing cells when mixed with untransformed wild-type cells. Clathrin-minus (A-D) or wild-type (E-G) ecmA-expressing cells were mixed with wild-type cells, allowed to develop, fixed, stained, and photographed. EcmA-expressing cells were scattered throughout mounds in both clathrin-minus (A) and wild-type (E) mixtures. At the finger stage, clathrin-minus ecmA-expressing cells were left behind at the base of the finger (B), while wild-type ecmA-expressing cells were primarily in the tip of the finger (F). In mature fruiting bodies, clathrin-minus ecmA-expressing cells were seen primarily at the base of the fruiting body in the basal disc and lower stalk (D) while some were also found in the lower cup (C). Wild-type ecmA-expressing cells were in the upper and lower cups and stalk (G). Scale bar, 50 μm.
the sorus, however, the ability of these cells to form functional spores remained uncertain. To examine this possibility, we measured the germination and survival rates of spores derived from mixing experiments. We mixed clathrin-minus cells (blasticidin-resistant) with wild-type cells (no antibiotic resistance). The mixtures were allowed to develop into mature fruiting bodies. Spores were harvested, treated with detergent to lyse undifferentiated amoebae and germinated in media containing blasticidin. All spores should germinate, but because only clathrin-minus spores carry antibiotic-resistance, only clathrin-minus cells should grow under selective pressure.

All mixtures formed fruiting bodies and virtually all spores from these fruiting bodies germinated (data not shown). No mutant cells survived selective pressure, demonstrating that clathrin-minus cells fail to form functional spores. In parallel experiments using control cells that were antibiotic-resistant, we recovered antibiotic-resistant amoebae even when only 10% of the cells in a mixture carried antibiotic resistance. This demonstrates that we were able to detect small numbers of antibiotic-resistant spores in this assay.

Sporulation is also known to be regulated by intracellular cyclic AMP signals (Kay, 1989). Thus it was possible that clathrin-minus cells were defective in this signaling pathway. Sporulation can be induced in vitro using the membrane-permeable cyclic AMP analog 8-bromo cyclic AMP in a low salt buffer (Kay, 1982). We were able to induce wild-type cells to form spores at a rate of 10-20% (data not shown) using 8-bromo cyclic AMP. Clathrin-minus cells never formed spores in these assays. Interpretation of the results of this assay is difficult because clathrin-minus cells swelled in the low osmotic spore induction buffer. This buffer is very specific for spore induction, and our attempts to modify the buffer to make it more hospitable for clathrin-minus cells resulted in a buffer that would not support spore induction of wild-type cells. Therefore, because of the osmotic sensitivity of clathrin-minus cells, we were not able to determine whether signaling necessary for sporulation was inducible using cyclic AMP analogs.

**DISCUSSION**

**Role of clathrin in early Dictyostelium development**

Our results show that Dictyostelium cells proceeded through early development even in the absence of clathrin heavy chain, although they were slower than wild-type cells. That is, clathrin-minus mutants acquired the shape changes required for chemotaxis, adopted the polarized morphology of a migrating cell and coordinated all of the signaling required to mobilize streams of cells toward a central aggregation point. Endocytosis has been proposed to play a central role in the removal of cell adhesion receptors from the leading edge of a polarized, migrating cell.
(Bretschger, 1984, 1988; Lawson and Maxfield, 1995). Endocytosis has also been proposed to be a mechanism for the sequestration of cyclic AMP receptors during down-regulation (Wang et al., 1988). Our finding that clathrin-minus cells moved toward an aggregation center argues either that the endocytosis of adhesion receptors is not required for the directed migration of Dictyostelium amoebae or that their internalization occurs through non-clathrin-mediated endocytosis. In addition, clathrin-coated vesicle traffic does not appear to be the primary mechanism for the down-regulation of cyclic AMP receptors from the surface of developing cells. While not required for early development, clathrin-mediated traffic may serve to increase the efficiency of cellular processes because both cell migration and the down-regulation of receptors was less efficient in the clathrin-minus mutants.

### Morphogenetic defects exhibited by clathrin-minus mutants

While clathrin-minus cells were able to construct mounds that were wild type in appearance, subsequent developmental morphogenesis was severely impaired. Terminal structures made by the mutant cells were highly dependent on environmental conditions and ranged from elongated finger-like projections to small fruiting bodies containing a rounded sorus on top of a rough stalk.

Proper development of a wild-type fruiting body requires that cells differentiate into stalk or spore cell types, and also that the differentiated cells sort to their appropriate location. The expression of wild-type levels of prestalk and prespore mRNAs in the mutant cells demonstrated that they received and responded correctly to signals for cell-type differentiation. Examination of the spatial distribution of prestalk and prespore cells, as revealed by promoter/lacZ reporter constructs, demonstrated that the differentiated cells failed to sort efficiently to their proper site. While some prestalk cells and prestalk cells migrated to their correct destination, many cells were scattered throughout the terminal structure. Even when mixed with an abundance of wild-type cells, most mutant prestalk and prespore cells incorporated inefficiently into the wild-type fruiting body and remained at the base of the structure. In contrast, wild-type prestalk and prestalk cells sorted exclusively to their proper destination. Thus, clathrin may play a role that serves to increase the efficiency of sorting during development. For example, the rapid clearance of plasma membrane proteins by clathrin-coated vesicles could facilitate cell surface changes and thereby increase the efficiency of cell-cell signaling mechanisms important for development.

### Requirement for clathrin in Dictyostelium spore-cell but not stalk-cell differentiation

Despite the inefficiency of sorting prestalk cells, clathrin-minus cells were striking in their ability to form an intact stalk. Even when terminal development resulted in a finger-like projection, clathrin-minus cells formed a functional stalk within the projection. These stalk cells were highly vacuolated and arranged in rows. This demonstrates that clathrin is not required for terminal differentiation or morphogenesis of stalk cells.

In contrast with their capacity to differentiate into stalk cells, clathrin-minus cells displayed a complete block in sporulation. The mutant cells did not express the sporulation-specific spiA gene product and also failed to acquire the distinct ellipsoidal shape of a differentiated spore cell. Even mixing the mutant cells with an abundance of wild-type cells did not create an environment permissive for sporulation of clathrin-minus cells. Because clathrin-minus cells formed one terminal cell-type but not another, we can rule out a pleiotropic defect in cell-type differentiation. This suggests that clathrin plays a specific role in spore cell differentiation.

The wealth of information about the specific role of clathrin in membrane traffic provides a framework for speculation about how clathrin functions in spore cell differentiation. In animal cells, clathrin-coated vesicles derived from the cell surface internalize receptors important for cell-cell communication. Examples of these receptors include the epidermal growth factor receptor, insulin receptor and β-adrenergic receptor (Brodsky, 1988; Zhang et al., 1996). By analogy, clathrin-coated vesicles in Dictyostelium cells could also mediate the internalization of a receptor required for sporulation. As with the animal cell receptors, this receptor could also trigger a signaling cascade that regulates sporulation.

Studies with animal cells have also shown that clathrin-coated vesicles derived from the trans-Golgi network transport lysosomal hydrolases (Kornfeld and Mellman, 1989). In Dictyostelium cells, clathrin functions in the transport and regulated secretion of lysosomal enzymes (Ruscetti et al., 1994). In a similar fashion, clathrin may mediate transport or secretory events required for development. For example, Dictyostelium spore formation requires the secretion of spore coat proteins from the prespore vesicle, a specialized compartment found only in prespore cells (West and Erdos, 1990). Perhaps clathrin-minus cells are unable to effect the trafficking of spore coat proteins to or from the prespore vesicle and are therefore unable to encapsulate themselves in a spore coat. More intensive studies of the clathrin-minus cells may pinpoint the precise role of clathrin in the differentiation of spore cells during Dictyostelium development.

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