

# Clathrin Heavy Chain Functions in Sorting and Secretion of Lysosomal Enzymes in *Dictyostelium discoideum*

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**Abstract.** The clathrin heavy chain is a major component of clathrin-coated vesicles that function in selective membrane traffic in eukaryotic cells. We disrupted the clathrin heavy chain gene (*chcA*) in *Dictyostelium discoideum* to generate a stable clathrin heavy chain-deficient cell line. Measurement of pinocytosis in the clathrin-minus mutant revealed a four- to five-fold deficiency in the internalization of fluid-phase markers. Once internalized, these markers recycled to the cell surface of mutant cells at wild-type rates. We also explored the involvement of clathrin heavy chain in the trafficking of lysosomal enzymes. Pulse chase analysis revealed that clathrin-minus cells processed most  $\alpha$ -mannosidase to mature forms, however, ~20–25% of the precursor molecules remained uncleaved, were missorted, and were rapidly secreted by the constitutive secretory pathway. The remaining in-

tracellular  $\alpha$ -mannosidase was successfully targeted to mature lysosomes. Standard secretion assays showed that the rate of secretion of  $\alpha$ -mannosidase was significantly less in clathrin-minus cells compared to control cells in growth medium. Interestingly, the secretion rates of another lysosomal enzyme, acid phosphatase, were similar in clathrin-minus and wild-type cells. Like wild-type cells, clathrin-minus mutants responded to starvation conditions with increased lysosomal enzyme secretion. Our study of the mutant cells provide in vivo evidence for roles for the clathrin heavy chain in (a) the internalization of fluid from the plasma membrane; (b) sorting of hydrolase precursors from the constitutive secretory pathway to the lysosomal pathway; and (c) secretion of mature hydrolases from lysosomes to the extracellular space.

**T**HE organized and directed movement of intracellular membranes and proteins are fundamental to the basic organization and functions of the eukaryotic cell. One way in which cells selectively invaginate and direct membrane traffic is via clathrin-coated regions of membranes and vesicles. Clathrin complexes on the plasma membrane gather specific receptors and facilitate receptor internalization into clathrin-coated vesicles for subsequent delivery to endosomes (Brodsky, 1988; Keen, 1990). As clathrin-coated vesicles bring receptors and their ligands into the cell, nutrients and fluids can follow by bulk-flow pinocytosis. Membrane proteins and extracellular liquids are also internalized by non-clathrin-mediated pathways; however, the overall contribution of this pathway to endocytosis is not known for most cells (Hansen et al., 1991). Previous studies of clathrin-deficient *Dictyostelium discoideum* cells created by antisense vectors demonstrated that the majority of fluid-phase endocytosis was dependent on the presence of functional clathrin (O'Halloran and Anderson, 1992a). However,

the low level of endocytosis exhibited by the antisense cells could be caused by a residual clathrin-mediated pathway and not by non-clathrin-mediated endocytosis.

Beyond its role in endocytosis, clathrin may also be involved in the sorting of lysosomal enzymes from the *trans*-Golgi network (TGN)<sup>1</sup> (Kornfeld and Mellman, 1989). Proteins destined for lysosomes are synthesized and modified as they travel the secretory pathway. In mammalian cells, the oligosaccharide side chains of lysosomal enzymes are modified by phosphorylation of mannose sugars, and these moieties are bound by the cation-dependent or the cation-independent mannose-6-phosphate receptor (MPR) (Kornfeld and Mellman, 1989). The MPR binds lysosomal enzymes in the TGN, and this receptor-ligand complex is targeted to the endolysosomal system. In a targeting process analogous to receptor-mediated endocytosis at the plasma membrane, MPRs and their lysosomal protein cargo may cluster in the TGN, associate with clathrin, and bud from

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1. *Abbreviations used in this paper:* chc, clathrin heavy chain; chc<sup>-</sup>, clathrin-minus *Dictyostelium* cell line; *chcA*, *chc* gene in *Dictyostelium*; Meses buffer, 20 mM MES (2-[*N*-morpholino]ethanesulfonic acid); MPR, mannose-6-phosphate receptor; TGN, *trans*-Golgi Network; 2H9, monoclonal antibodies against  $\alpha$ -mannosidase.

the membrane as clathrin-coated vesicles. This trafficking mechanism is supported by studies in mammalian cells that have shown that lysosomal enzymes are found in clathrin-coated vesicles, and that the cation-independent MPR can be associated with clathrin coats through its COOH-terminal cytoplasmic tail. Thus, clathrin may be a key component in the MPR-dependent sorting process (Dahms et al., 1989; Lobel, 1989).

*Dictyostelium* and the budding yeast *Saccharomyces cerevisiae* apparently lack MPRs and lysosomal enzyme targeting proceeds along an MPR-independent pathway (Cardelli et al., 1986, 1987; Valls et al., 1987). This MPR-independent pathway may also exist in mammalian cells (Dahms et al., 1989). To date, the mechanism of protein sorting in the MPR-independent pathway is not well understood. Indeed, membrane proteins in yeast may not require specialized vesicles for transit to the vacuole because recent evidence suggests that the vacuole may be the default compartment (Roberts et al., 1992). *S. cerevisiae* mutants that carry a temperature-sensitive allele of the clathrin heavy chain show an initial inhibition of protein sorting to the lysosome-like vacuole when incubated at the nonpermissive temperature for short periods. However, this effect is only transient; over long periods at the nonpermissive temperature, these mutants recover the ability to target proteins to the vacuole, suggesting that clathrin is not an absolute requirement for protein targeting to the vacuole (Seeger and Payne, 1992a).

Like *S. cerevisiae*, *Dictyostelium* apparently uses a MPR-independent pathway to target hydrolases to lysosomes (Cardelli et al., 1993). *D. discoideum* lysosomal hydrolases are synthesized as membrane-bound precursor polypeptides in the ER, where they are glycosylated on Asn residues (Cardelli et al., 1986, 1987). The precursor polypeptides are transported to the Golgi apparatus, where the oligosaccharide side chains are further modified by sulfation and phosphorylation (Freeze and Wolgast, 1986; Mierendorf et al., 1985). In a later Golgi compartment or in endosomes, the precursors are cleaved to generate intermediate subunits, and finally in the lysosome, they are processed into the soluble mature enzymes (Cladaras and Kaplan, 1984; Richardson et al., 1988). The lysosome is not a dead-end compartment for the newly synthesized mature lysosomal hydrolases; they are secreted from the cell along a specific pathway thought to include a distinct intracellular compartment (termed the postlysosomal compartment) (Padh et al., 1993).

We have chosen to examine the role of the clathrin heavy chain in two aspects of membrane traffic: the endocytosis of fluid from the plasma membrane and the trafficking of lysosomal hydrolases to lysosomes. As a tool for these studies, we have generated a clathrin-minus *Dictyostelium* cell line (*chc*<sup>-</sup>) that lacks a functional clathrin heavy chain gene. We find that the *chc*<sup>-</sup> cells are viable; however, the mutant cells are defective in fluid-phase endocytosis, sorting of hydrolases to the lysosome, and secretion of hydrolases from the lysosome.

## Materials and Methods

### Cell Growth

*D. discoideum* (strain Ax2) was grown in HL5 medium (1% oxidized proteose

peptone, 1% glucose, 0.5% yeast extract (Difco Laboratories, Inc., Detroit, MI), 2.4 mM Na<sub>2</sub>HPO<sub>4</sub>, and 8.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 6.5) supplemented with penicillin-streptomycin (GIBCO BRL, Gaithersburg, MD) at 60 U/ml. For transformant selection, the media also contained 10 µg/ml G418 (GIBCO BRL). In all other procedures, cells were grown in T<sub>25</sub> tissue culture flasks in HL5 media unless otherwise indicated.

### Generation and Characterization of *chc*<sup>-</sup> Cell Line

Standard molecular biology techniques were performed according to the methods of Sambrook et al. (1989). pTO123 was created by replacing the middle portion of the clathrin heavy chain gene with the 2.1-kb neomycin resistance gene. The plasmid pTO123 was constructed from pTZ2.1 (Manstein et al., 1989) with the 2.1-kb neomycin selectable marker of pTZ2.1 flanked 5' by the 2.8-kb BamHI-BglII fragment of the *chcA* gene (O'Halloran and Anderson, 1992b) inserted into the BamHI site and 3' by the 1.1-kb SspI fragment cloned into the Sall site (blunted) of pTZ2.1.

pTO123 was transformed into Ax2 cells by electroporation using a gene pulser (Bio Rad Laboratories, Richmond, CA) as according to Kuspa and Loomis (1992). Briefly, Ax2 cells ( $2 \times 10^7$ ) were pelleted by centrifugation at 228 g and resuspended in 0.8 ml electroporation buffer (10 mM sodium phosphate, pH 6.1, and 50 mM sucrose). Plasmid DNA (15 µg) was mixed with the resuspended cells in a plastic 0.5-cm cuvette and incubated on ice for 10 min. Electroporation was carried out on the cells at 1.2 kV, 3.0 µF, and 200 Ω, followed by a 10-min recovery period on ice. The cells then recovered overnight in a petri dish containing HL5/Pen-Strep/G418 and were plated the next day into three 96-well plates.

*Dictyostelium* DNA was prepared by Sarkosyl lysis followed by organic extraction (Manstein et al., 1989). Southern blots were prepared from 0.7% agarose gels and transferred to Hybond N+ (Amersham Corp., Arlington Heights, IL) using standard methods (Sambrook et al., 1989). RNA was prepared by the method of Nellen et al. (1987). For Northern blots, 10 µg of RNA was electrophoresed in a 1% agarose gel in the presence of 3% formaldehyde. After electrophoresis, equivalent bands of ribosomal RNA were seen in each sample of the ethidium bromide-stained gel, confirming equal loading of each RNA sample. Southern and Northern blots were probed with 10<sup>6</sup> cpm/ml of various *Dictyostelium* clathrin heavy chain DNA fragments labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using a random-primed DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN).

### Protein Characterization

For Western analysis, 100 µg of protein was analyzed on 7% SDS-polyacrylamide gels and subsequently transferred to nitrocellulose. The nitrocellulose was incubated with Blotto (5% dry milk, 0.2% Tween-20, 50 mM Tris-HCl, and 0.15 M NaCl, pH 7.5) and then incubated for 1 h with the rabbit anti-*Dictyostelium* clathrin heavy chain serum diluted 1:1000 in primary antibody solution (5% bovine serum albumin, 0.02% sodium azide, 50 mM Tris-HCl, and 0.15 M NaCl, pH 7.5). After washing for 1 h with 1× TBS (10 mM Tris, 150 mM NaCl, pH 7.4) containing 0.1% Triton X-100, the nitrocellulose was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) (Bio-Rad Laboratories) diluted 1:1000 in Blotto. After an additional hour of washes, the color was developed using 3 mg/ml 4-chloro 1-naphthol and hydrogen peroxide. Protein concentrations were determined with protein assay (Bio-Rad Laboratories) using bovine gamma globulin as a standard.

### Measurement of Pinocytosis

Pinocytosis was measured according to the method of Klein and Satre (1986). Log-phase cells were harvested, plated at  $1-2 \times 10^6$  cells/ml in HL5 (2 ml total) in a 60-mm petri dish, and grown overnight until the cells were at a titer of  $3-4 \times 10^6$  cells/ml. FITC-dextran (70,000 *M*<sub>r</sub>; Sigma Immunochemicals, St. Louis, MO) was added to a final concentration of 2 mg/ml. All samples were kept covered at 20°C, and zero time points were kept covered on ice water for 30 min before assaying. To stop pinocytosis, the cells were washed twice by centrifugation with ice-cold HL5 and resuspended in 10 ml ice-cold HL5. Cells were spun cold at 228 g for 5 min and the supernatant was removed. The cells were resuspended in 3 ml of 50 mM Na<sub>2</sub>HPO<sub>4</sub> buffer, pH 9.2, counted, and then lysed with 0.2% Triton X-100. Fluorescence was measured on a fluorimeter (Beckman Instruments, Inc., Fullerton, CA) using excitation and emission wavelengths of 470 and 520 nm, respectively. FITC-dextran was diluted to make a standard curve and the amount of FITC-dextran pinocytosed per 10<sup>6</sup> cells was calculated.

## Enzyme Assays

$\alpha$ -Mannosidase,  $\beta$ -glucosidase, and acid phosphatase enzyme activity were measured as previously described (Bennet and Dimond, 1986; Free and Loomis, 1974; Dimond and Loomis, 1974).

## Radiolabel Pulse Chase Analysis and Immunoprecipitation

Cells were grown to ~80–90% confluency in 10 ml of HL5 in T<sub>25</sub> flasks. The media was gently aspirated from each plate, replaced with 2 ml HL5 containing 625  $\mu$ Ci of [<sup>35</sup>S]methionine (6,000  $\mu$ Ci/ml; New England Nuclear, Du Pont Chemicals, Boston, MA), and incubated at 21°C for 20–30 min. At the end of the pulse period, the labeled media was collected and replaced by 2 ml of unlabeled HL5. For every chase point, one flask was harvested by gently removing the media and resuspending the cell monolayer in 1 ml of 0.5% Triton X-100. The cell and media samples were adjusted to a final concentration of 1 $\times$  C buffer (50 mM Na<sub>2</sub>EDTA, 150 mM NaCl, 50 mM Tris Base, 0.5% NP-40, 2 mM methionine, 1 mM NaN<sub>3</sub>) using a 5 $\times$  concentrated stock. The samples were precleared using 0.1 ml of 1 $\times$  C-buffered Pansorbin (Calbiochem-Novabiochem Corp., La Jolla, CA); the samples were incubated on ice for 1 h and then centrifuged at 12,000 g for 3 min to pellet the Pansorbin. The supernatant was incubated on ice for 1.5–2 h with 1  $\mu$ l of ascites fluid containing monoclonal antibodies against  $\alpha$ -mannosidase. 125  $\mu$ l of 1 $\times$  C-buffered Pansorbin were added to the samples and then incubated on ice for 1–1.5 h. The Pansorbin/antibody/antigen complexes were washed by centrifugation in 1 $\times$  C three times and eluted in 2 $\times$  Laemmli buffer by heating for 3–5 min at 80°C. Samples were run on 7.5% SDS-PAGE gels (Laemmli, 1980), subjected to fluorography (EN<sup>3</sup>HANCE; New England Nuclear), and exposed using x-ray film (X-Omat; Eastman Kodak Co., Rochester, NY).

## Percoll Gradient Fractionation

Cells were grown in 10 ml HL5 medium in T<sub>25</sub> flasks to 80–90% confluency, mechanically harvested, and added to 250 ml of G<sub>M1</sub> carrier cells growing at 3  $\times$  10<sup>6</sup> cells/ml (see Cardelli et al., 1990). The strain G<sub>M1</sub> contains mutations in the  $\alpha$ -mannosidase and  $\beta$ -glucosidase genes and is effectively devoid of both of these enzymatic activities. Cells were washed twice by centrifugation (1,000 g for 5 min) and resuspended in Meses buffer (20 mM MES (2-[N-morpholino]ethanesulfonic acid), 1 mM EDTA, and 250 mM sucrose, pH 6.5). The cells were mechanically disrupted using a tight-fitting Dounce homogenizer (Wheaton Scientific, Millville, NJ). The unbroken cells and nuclei were pelleted by 1,000 g centrifugation, and the postnuclear supernatant was recovered. The unbroken cells were resuspended in 3 ml Meses buffer and subjected to a second round of homogenization and centrifugation. The post-nuclear supernatants were combined and 5 ml were layered on a cushion of 24% Percoll in buffer containing 250 mM sucrose. The gradients were formed in a fixed-angle rotor (type 40.2) at 25,000 g for 1 h at 4°C. 1.25-ml fractions were collected starting from the bottom of the gradient, and the membranes were solubilized by the addition of 10% Triton X-100 to a final concentration of 0.5%. Enzymes were assayed as described above.

## Immunofluorescent Cell Staining

Immunofluorescent cell staining was performed as previously described (Bush and Cardelli, 1989) with minor modifications. Cells were grown in 10-ml HL5 media in T<sub>25</sub> flasks to 50% confluency. The cells were mechanically harvested and 300  $\mu$ l of cells were pipetted onto glass coverslips inside a covered 35  $\times$  10 mm plastic petri dish and were allowed to adhere for 1 h. The cells were fixed in 2.5 ml of 3.7% formaldehyde in PBS (10 mM Na<sub>2</sub>PO<sub>4</sub>, 150 mM NaCl, pH 7.4) for 1 h at room temperature. The fixative was removed from the petri dishes, and the coverslips were bathed in a blocking solution (PBS with 0.1% gelatin) for 10 min followed by three washes in 2.5 ml PBS supplemented with 0.5% Tween 20. Monoclonal antibodies against  $\alpha$ -mannosidase (2H9) were added to the top of the coverslips at a 1:100 dilution in PBS containing 0.1% saponin and 2.5 mg/ml BSA (antibody solution). After a 2-h incubation at 4°C, the coverslips were washed three times as described above. Fluorescein-conjugated goat anti-mouse secondary antibodies (Southern Biotech, Birmingham, AL) were added to the coverslips at a 1:50 dilution in antibody solution and incubated at 4°C for 1.5 h. The coverslips were washed as described above and mounted to microscope slides using PBS-buffered glycerol supplemented with 1 mg/ml phenylenediamine (Johnson, 1981) and sealed with clear nail polish. Photographs were taken with black and white 400 ASA T-max film.

## Standard Secretion Assays

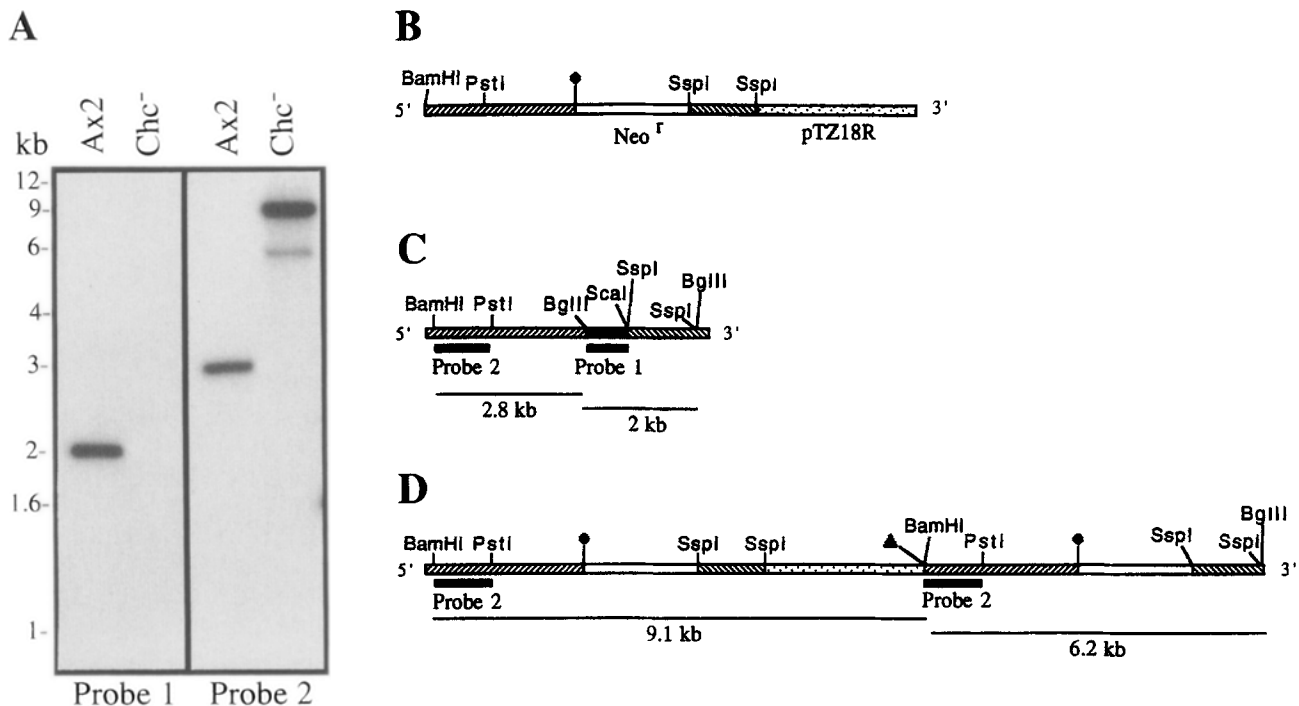
Cells were grown at 21°C in 10 ml HL5 media on T<sub>25</sub> tissue culture flasks until they were ~70–90% confluent. The media were gently aspirated, and 2 ml of HL5 media or starvation buffer (10 mM phosphate buffer, pH 6.5, 50 mM glucose) were added to each plate. At the times indicated, one T<sub>25</sub> plate was harvested by gentle aspiration of the media or buffer, and the attached cell monolayer was harvested by addition of 0.5 ml of 0.5% Triton X-100.

## Results

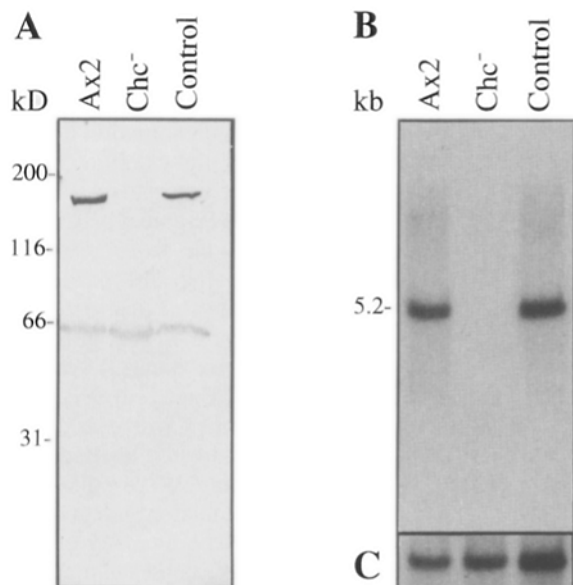
### Creation and Characterization of Clathrin-minus Cells

The clathrin heavy chain (*chc*) gene in *Dictyostelium*, *chcA*, exists as a single copy 5.3-kb gene (O'Halloran and Anderson, 1992b) (Figure 1 C). To establish a permanent clathrin-minus cell line, we targeted the *chcA* gene with a linear piece of DNA designed to replace 0.8 kb from the middle portion of the gene with a 2.2-kb selectable neomycin resistance marker. To accomplish homologous recombination of the marker into the *chcA* locus, the neomycin marker was flanked by 2.8 kb of coding sequence from the 5' region of the gene and 1.2 kb of sequence from the 3' end of the gene (Fig. 1 B). This linear DNA construct was introduced into wild-type Ax2 cells by electroporation. The transformation procedure yielded a total of 41 independent G418-resistant colonies that were analyzed for the presence of clathrin heavy chain protein by Western blot analysis. These tests revealed one colony, HO101, that was devoid of the 190-kD clathrin heavy chain protein (Fig. 2 A). We selected the HO101 colony for further study, as well as another colony, HO102, with wild-type levels of clathrin heavy chain to use as a control. Northern blot analysis of the HO101 cells demonstrated that the absence of *chc* protein was caused by a lack of *chc* mRNA. While cells from the control colony had levels of clathrin heavy chain message that were equivalent to the parental strain Ax2, the *chc*<sup>-</sup> strain had a complete absence of the 5.2-kb clathrin heavy chain message (Fig. 2 B).

Southern blots of genomic DNA confirmed that the mutant *chc*<sup>-</sup> cell line arose from homologous recombination of the selectable marker into the *chcA* locus. A probe derived from the portion of the gene that was replaced by the selectable marker detected the 2.8-kb BamHI-BglII fragment predicted in wild-type DNA, but detected no signal from genomic DNA purified from the mutant cell line (Fig. 1 A, probe I). This result confirmed that the *chcA* gene was destroyed by the replacement of the middle portion of the *chcA* gene by the neomycin marker in a double crossover event. A second probe derived from the 5' end of the *chcA* gene revealed the predicted 2.8-kb BamHI-BglII fragment in wild-type Ax2 DNA, but detected a 9.1 kb BamHI and a 6.2-kb BamHI-BglII band in the mutant DNA. This pattern can be explained by extra copies of the parent plasmid in tandem integration into the 5' end of the gene (Fig. 1 D). The intensity of the 9.1-kb BamHI band suggested that multiple copies of the parent plasmid integrated into the gene, a phenomenon that has been observed previously in *Dictyostelium* transformants selected with neomycin (Manstein et al., 1989). Furthermore, the presence of the 9.1-kb BamHI band demonstrated that the plasmid had integrated exclusively into the *chcA* locus; no bands attributable to other loci were detected.



**Figure 1.** *Chc*<sup>-</sup> cells contain a disrupted clathrin heavy chain gene. (A) Southern blots of genomic DNA from Ax2 and *chc*<sup>-</sup> cells were digested with BamHI and BglII and probed with the 0.8-kb BglII-ScaI fragment (*probe 1*) and the 1.1-kb BamHI-PstI fragment (*probe 2*) of the *chcA* gene. (B) The linearized plasmid, pTO123, used for homologous recombination, contains the *chcA* gene in pTZ18R in which the 0.8-kb BglII-ScaI *chcA* fragment was replaced with the 2.2-kb neomycin resistance marker. (C) The wild-type *chcA* restriction map is shown. Probe 1 detects a 2.0-kb fragment in Ax2 DNA while probe 2 detects a 2.8-kb fragment. (D) The restriction map of *chc*<sup>-</sup> DNA is shown. Probe 1 does not detect any fragment in *chc*<sup>-</sup> DNA, indicating that this portion of the *chcA* gene has been deleted from the mutant DNA. Probe 2 detects 6.2- and 9.1-kb bands in *chc*<sup>-</sup> DNA, indicating that the plasmid (9 kb) has integrated into the genome and disrupted the *chcA* gene, yielding a 6-kb fragment. The triangle represents the site where multiple copies of the 9-kb plasmid have integrated into the genome. Stippled box, pTZ18R; white box, neomycin resistance gene; hatched boxes, *chcA* gene.

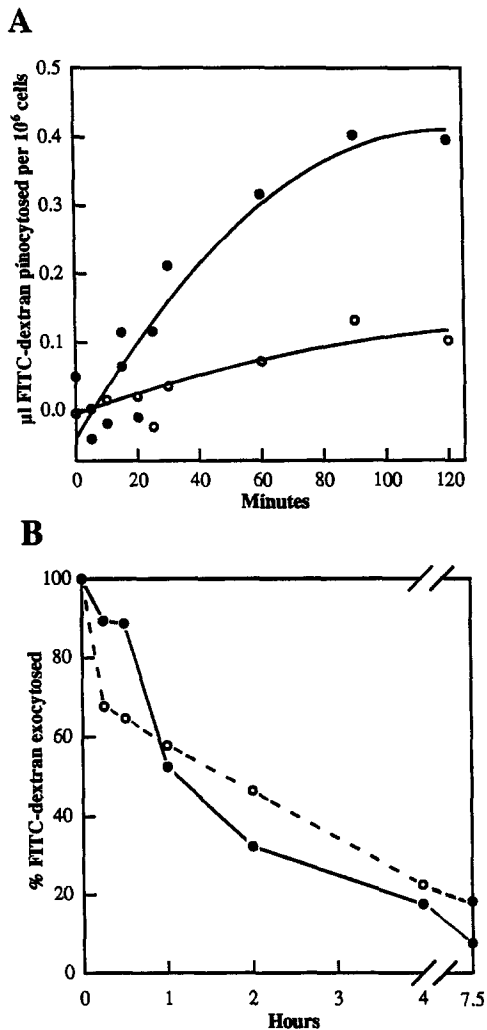


**Figure 2.** Clathrin heavy chain protein and RNA are undetectable in *chc*<sup>-</sup> cells. (A) Whole-cell lysates were separated on a 7.5% acrylamide gel, transferred to nitrocellulose, and clathrin heavy chain was detected with a rabbit anticlathrin polyclonal antibody followed by HRP-conjugated goat anti-rabbit secondary antibody. (B) Total RNA was isolated, separated on a formaldehyde gel, transferred to nitrocellulose, and probed with probe 1. Ax2 and control cells show the expected 5.2-kb RNA signal, but *chc*<sup>-</sup> cells show no signal, indicating that the *chcA* RNA is not present. (C) The same blot was stripped of the *chcA* probe and hybridized with a myosin heavy chain probe to confirm equal loading.

### Endocytosis by *chc*<sup>-</sup> Cells

We found that the *chc*<sup>-</sup> cell line was viable but grew more slowly than control or wild-type cultures. Exponentially growing cultures of control cell lines had a doubling time of 12 h, whereas exponentially growing cultures of *chc*<sup>-</sup> cells doubled every 21 h. Since *Dictyostelium* cells grow in axenic media by virtue of fluid-phase endocytosis, the slow growth of the *chc*<sup>-</sup> cells could be explained by a defect in endocytosis. To evaluate the role of the clathrin heavy chain in fluid-phase endocytosis, we measured the ability of the *chc*<sup>-</sup> cells to internalize the fluid phase marker, fluorescein-labeled dextran. This 70,000-*M<sub>r</sub>* molecule is too large to cross the membrane passively and thus serves as a fluid phase marker. Logarithmically growing cultures of control cells and *chc*<sup>-</sup> cells were incubated in growth media with the labeled dex-

Results show that the 190-kD clathrin heavy chain protein is present in Ax2 and control cells, but is absent from *chc*<sup>-</sup> cells. The band at 60 kD is nonspecific but serves as a convenient loading control. (B) Total RNA was isolated, separated on a formaldehyde gel, transferred to nitrocellulose, and probed with probe 1. Ax2 and control cells show the expected 5.2-kb RNA signal, but *chc*<sup>-</sup> cells show no signal, indicating that the *chcA* RNA is not present. (C) The same blot was stripped of the *chcA* probe and hybridized with a myosin heavy chain probe to confirm equal loading.



**Figure 3.** Pinocytosis in *chc<sup>-</sup>* cells is defective but exocytosis is normal. (A) Control (closed circles) or *chc<sup>-</sup>* cells (open circles) were incubated with 2 mg/ml FITC-dextran. Intracellular fluorescence was measured at the time points indicated, and the amount of FITC-dextran internalized per 10<sup>6</sup> cells was plotted. Control and *chc<sup>-</sup>* cells pinocytosed a maximum of 0.4  $\mu$ l and 0.1  $\mu$ l dextran per 10<sup>6</sup> cells, respectively. The initial rate of dextran uptake in *chc<sup>-</sup>* cells was 4.6-fold less than control cells. (B) Control (closed circles) or *chc<sup>-</sup>* cells (open circles) were incubated with 2 mg/ml FITC-dextran for 2 h, washed twice with fresh HL5, and incubated with fresh HL5 for the time points indicated. Intracellular fluorescence was measured and the percent of the total FITC-dextran exocytosed was plotted. *Chc<sup>-</sup>* and control cells exocytosed FITC-dextran at similar rates.

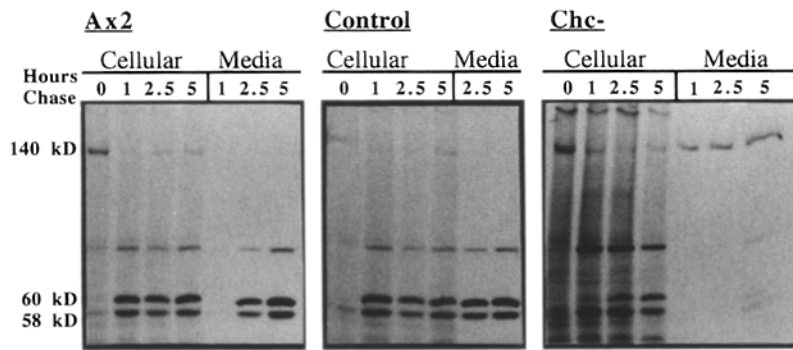
trian for various amounts of time. Subsequently, the cells were washed free of extracellular dextran, lysed with detergent, and the amount of internalized dextran was quantified with a fluorimeter. In comparison with control cells, *chc<sup>-</sup>* cells showed a marked depression of both the rate and the extent of fluid-phase endocytosis (Fig. 3 A). *Chc<sup>-</sup>* cells accumulated the fluid-phase marker at a rate that was 4.6-fold lower than control cells (1.4 nl dextran/10<sup>6</sup> cells per min vs 6.5 nl dextran/10<sup>6</sup> cells per min, respectively, for the first 30 min). The extent to which the mutant *chc<sup>-</sup>* cells accu-

mulated the marker was also depressed relative to control cells: uptake reached equilibrium at levels fourfold lower than wild-type cells (0.1  $\mu$ l dextran/10<sup>6</sup> cells vs 0.4  $\mu$ l dextran/10<sup>6</sup> cells). These results are in agreement with previous results with clathrin-minus cells generated by antisense plasmids that showed initial rates eightfold lower than wild type and a fourfold lower accumulation of fluid-phase markers (O'Halloran and Anderson, 1992a).

The low rate of accumulation of dextran by the *chc<sup>-</sup>* cells suggests that clathrin acts directly to facilitate endocytosis from the plasma membrane. However, an alternative scenario could also explain the reduced rate and extent of fluid phase uptake by the *chc<sup>-</sup>* cells. It is possible that the fluid phase markers are endocytosed at normal levels but that they recycle to the media by a faster rate of exocytosis. This result would be observed if, for example, clathrin was required for the presence of an endosomal compartment where the pinocytosed material is accumulated. To distinguish between these possibilities, we examined exocytosis of fluorescein-labeled dextran in *chc<sup>-</sup>* and control cells. The cells were loaded with dextran for 2 h, assuring that their endocytic compartments were filled completely. We then placed the cells in fresh media, and measured the amount of dextran that remained in the cell over time. Fig. 3 B shows that both the rate and extent of exocytosis of internalized dextran was similar: control cells exocytosed 0.79% of their dextran stores/min while the mutants exocytosed 0.70%/min (averaged during 60 min). Thus, the failure of *chc<sup>-</sup>* mutants to accumulate dextran can be attributed to a deficiency in internalization and not increased export of internalized fluid to the extracellular space.

#### **The Clathrin Heavy Chain is Required for Efficient Protein Sorting of Lysosomal $\alpha$ -Mannosidase**

In addition to its role in endocytosis, clathrin may be involved in sorting of proteins from the secretory pathway (Kornfeld and Mellman, 1989; Seeger and Payne, 1992a). To determine the role of the clathrin heavy chain in the sorting of *D. discoideum* lysosomal proteins, we performed radiolabel pulse-chase analysis to examine the processing and secretion of the lysosomal enzyme,  $\alpha$ -mannosidase, in control and *chc<sup>-</sup>* cells.  $\alpha$ -Mannosidase is synthesized as a 140-kD membrane-bound precursor that is processed to an 80-kD intermediate and the 58-kD mature subunit in the late Golgi or early endosomes (Cardelli et al., 1990). In lysosomes, the 80-kD intermediate is cleaved to generate the 60-kD mature subunit (Richardson et al., 1988) and together, the 58- and 60-kD subunits form the soluble heterotetrameric holoenzyme found in lysosomes. Normally,  $\geq 95\%$  of the  $\alpha$ -mannosidase precursor is segregated from the constitutive secretory pathway into the lysosomal targeting pathway, while the remaining precursor polypeptides continue in the default secretory pathway and are rapidly secreted from the cell in precursor form (Cardelli et al., 1986; Mierendorf et al. 1985). Logarithmically growing cultures of Ax2, control and *chc<sup>-</sup>* cells were metabolically labeled for 40 min with <sup>35</sup>S[methionine] (pulse) and then chased in unlabeled media for 5 h. Samples were taken at 0, 1, 2.5, and 5 h of chase and separated by centrifugation into cellular and supernatant fractions. Antibodies specific for  $\alpha$ -mannosidase were used to immunoprecipitate the enzyme from each



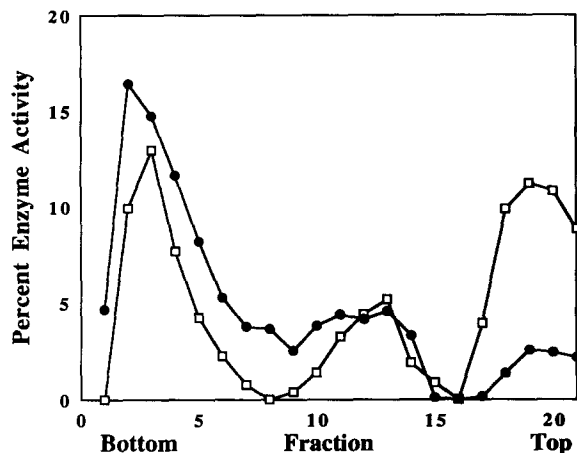
**Figure 4.** Pulse chase analysis of *chc*<sup>-</sup> cell lines. Growing cells were pulsed with [<sup>35</sup>S]methionine in HL5 media for 40 min and chased for the times indicated in unlabeled media. Each sample was incubated with monoclonal antibodies specific for  $\alpha$ -mannosidase. SDS-PAGE and fluorography were used to separate and visualize the immunoprecipitated protein.

sample (Fig. 4). The relative rates of synthesis of  $\alpha$ -mannosidase were 10-fold lower in *chc*<sup>-</sup> cells compared to wild-type and control cells (results not shown) and, therefore, the autoradiographs of the *chc*<sup>-</sup> cell samples required a longer exposure to achieve a signal comparable to Ax2 and control samples. We determined that the decreased rate of  $\alpha$ -mannosidase synthesis in *chc*<sup>-</sup> cells was the result of a decrease in the relative levels of  $\alpha$ -mannosidase message (data not shown). This may reflect the inability of *chc*<sup>-</sup> cells to detect or effectively secrete the prestarvation factor; a glycoprotein responsible for increasing transcription of the  $\alpha$ -mannosidase gene and other prestarvation factor-responsive genes (Schatzle et al., 1993). In all three strains examined,  $\alpha$ -mannosidase was synthesized as 140-kD precursor (Fig. 4, lanes marked 0). In each strain, ~90% of the intracellular precursor molecules were proteolytically processed into the 80-kD intermediate and 60- and 58-kD mature subunits by 1 h of chase (Fig. 4). By 5 h of chase, 80–90% of the cell-associated immunoprecipitable  $\alpha$ -mannosidase was found as the mature enzyme. In samples from control and Ax2 cells, <5% of the detectable  $\alpha$ -mannosidase precursor molecule was found extracellularly, and >90% of the intracellular precursor was processed into mature enzyme. Although *chc*<sup>-</sup> cells proteolytically processed most of the  $\alpha$ -mannosidase precursor, ~25–30% of the precursor remained unprocessed and was rapidly secreted (missorted) from the mutant cells (Fig. 4, *chc*<sup>-</sup> panel). Missorting of the newly synthesized  $\alpha$ -mannosidase precursor in the absence of clathrin heavy chain supports a role for clathrin in maintaining the fidelity of the sorting process.

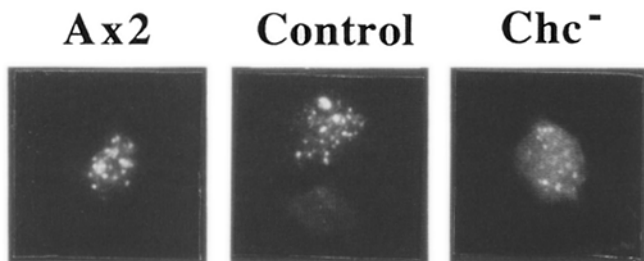
#### ***Lysosomal Enzymes Retained in chc<sup>-</sup> Cells are Correctly Localized to Lysosomes***

Three lines of evidence support the hypothesis that intracellularly retained  $\alpha$ -mannosidase was correctly targeted to lysosomes in *chc*<sup>-</sup> cells. First, the final processing event of the  $\alpha$ -mannosidase precursor molecule is the cleavage of the 80-kD intermediate polypeptide into the 60-kD mature subunit and this event occurs in lysosomes (Wood and Kaplan, 1985; Richardson et al., 1988; Mierendorf et al., 1985). Radiolabel pulse chase analysis (described above) revealed that *chc*<sup>-</sup> cells were capable of this final processing event suggesting that  $\alpha$ -mannosidase was localized to lysosomes. In addition, we performed subcellular fractionation to determine the localization of  $\alpha$ -mannosidase in the mutant cells. Control and *chc*<sup>-</sup> cells were broken in isotonic buffer and cell nuclei were pelleted. The remaining cellular or-

ganelles and cytoplasm were separated by virtue of their density on isopycnic Percoll gradients. Each fraction of the gradient was assayed for  $\alpha$ -mannosidase enzyme activity, which was expressed as the percent of total enzyme activity in the entire gradient (Fig. 5). Fractionation of control cells revealed a peak of  $\alpha$ -mannosidase enzyme activity predominantly in the dense fractions of the gradient (fractions 2–4), corresponding most likely to mature dense lysosomes. A second, smaller peak of enzyme activity was observed in less dense fractions (fractions 11–14), and it corresponded in density to ER and Golgi membranes (data not shown). These lighter membranes containing  $\alpha$ -mannosidase enzyme activity may be analogous to endosomal vesicles or transport vesicles carrying hydrolases to lysosomes. Fractions 16–21 represent the soluble portion of the gradient and enzyme activity in these fractions represents hydrolase-containing vesi-



**Figure 5.** Subcellular fractionation of *chc*<sup>-</sup> cells. Logarithmically growing cells were disrupted and separated by density on an isopycnic Percoll gradient. The fractions of the gradient were solubilized and assayed for enzyme activity. Fraction 1 represents the bottom of the gradient and the most dense fraction. The peak of  $\alpha$ -mannosidase enzyme activity in both *chc*<sup>-</sup> (●) and control (□) cells is found in fractions 2–5, presumably representing dense lysosomes. There is a smaller peak of enzyme activity in lighter membrane fractions (10–15) that correspond to the ER and Golgi fractions (data not shown). Fractions 16–21 represent the soluble portion of the gradient, and enzyme activities in these fractions may identify enzymes that were released from lysosomes during the fractionation process.



**Figure 6.**  $\alpha$ -Mannosidase is localized to punctate structures by immunofluorescent cell staining. Growing cells were allowed to adhere to coverslips, fixed with 3.7% formaldehyde in PBS, and permeabilized with saponin. Murine monoclonal antibodies specific for  $\alpha$ -mannosidase (2H9) were incubated with the fixed cells, and the bound antibodies were visualized using FITC-conjugated anti-mouse antibodies. The antibodies predominantly decorated distinct punctate structures in wild-type, control, and  $chc^-$  cells, suggesting the  $\alpha$ -mannosidase was localized to lysosomes.

cles that have been disrupted by the fractionation process. The overall profile on Percoll gradients of  $\alpha$ -mannosidase enzyme activity from  $chc^-$  postnuclear supernatants was very similar to that observed for control cells, except that a higher percentage of the total  $\alpha$ -mannosidase enzyme activity was found in the dense fractions. Furthermore, the peak of enzyme activity from fractionated  $chc^-$  cells was found in a slightly more dense fraction of the gradient than was the peak activity from control cells (Fig. 5, fraction 2 vs fraction 3). The relative levels of  $\alpha$ -mannosidase enzyme activity in the lighter membrane fractions were equivalent in both  $chc^-$  and control cells.

We also used indirect immunofluorescent cell staining to localize  $\alpha$ -mannosidase in cells. As indicated in Fig. 6,  $\alpha$ -mannosidase antibodies stained punctate intracellular structures consistent with lysosomal staining in each of the cell lines tested. Antibodies to lysosomal enzyme, acid phosphatase, also decorated punctate structures in  $chc^-$ , Ax2, and control cells (data not shown). Taken together, these data suggest that the clathrin heavy chain does not affect the localization of intracellularly retained lysosomal enzymes to dense lysosomes.

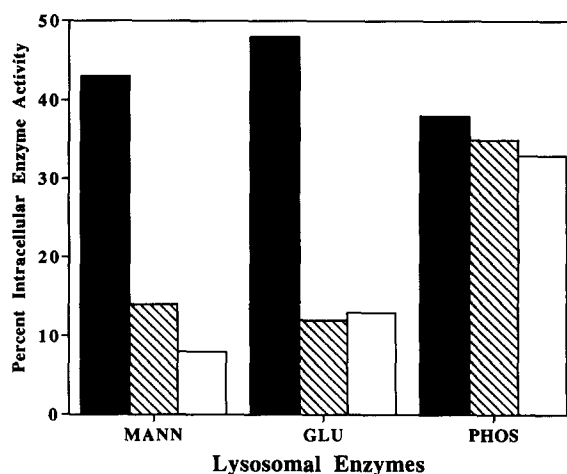
#### Secretion of Lysosomal Enzymes is Impaired in Clathrin Heavy Chain-deficient Cells

As indicated in Fig. 4, after 2.5 h of chase, the newly labeled intermediate and mature forms of  $\alpha$ -mannosidase were detected in the extracellular media in cultures of Ax2 and control cells, and after 5 h of chase,  $\sim 70\%$  of the radiolabeled mature  $\alpha$ -mannosidase had been secreted (Fig. 4, *Control* and *Ax2*). In contrast, secretion of the radiolabeled mature and intermediate forms of  $\alpha$ -mannosidase was severely impaired in  $chc^-$  cultures;  $<10\%$  of these radiolabeled forms were detected in the extracellular media after 5 h of chase (Fig. 4, *chc^-* panel).

To determine if hydrolases accumulated to higher levels in  $chc^-$  cells compared to wild type, we determined the steady-state intracellular versus extracellular distribution of three lysosomal enzymes,  $\alpha$ -mannosidase,  $\beta$ -glucosidase, and acid phosphatase. Logarithmically growing cultures (midlog phase) were separated into intracellular and media

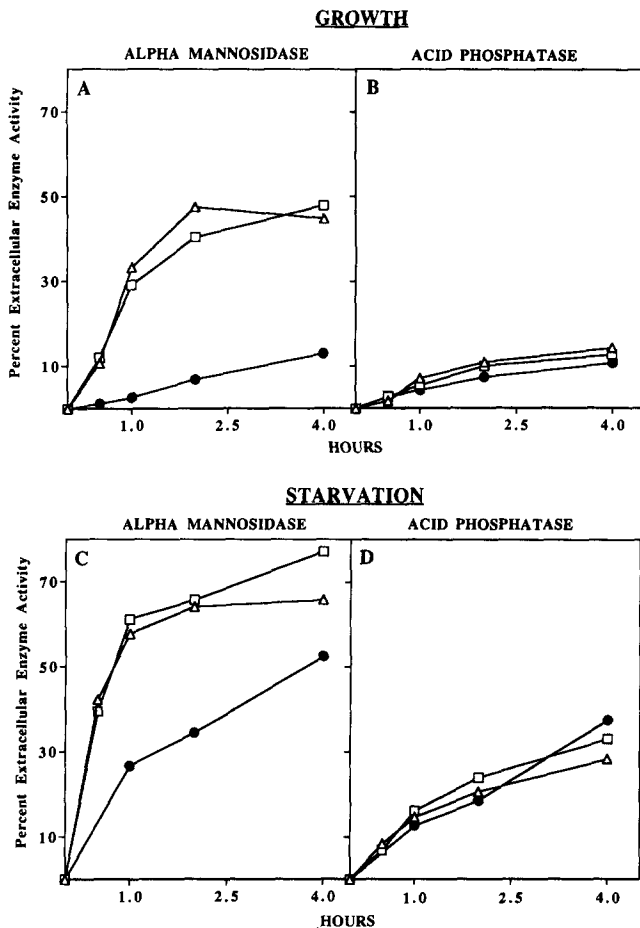
(extracellular) fractions by centrifugation. The intracellular enzyme activities of the three lysosomal hydrolases were measured and expressed as the percent of the total (intracellular and extracellular) enzyme activity (Fig. 7). Only 10–15% of the total  $\alpha$ -mannosidase and  $\beta$ -glucosidase enzyme activities was found in logarithmically growing wild-type and control cells, while in contrast, 45–50% of these glycosidases were retained in the  $chc^-$  cells. This fourfold increase in the level of intracellular lysosomal enzyme activity in  $chc^-$  cells is consistent with a secretion defect for  $\alpha$ -mannosidase and  $\beta$ -glucosidase. However, the amount of acid phosphatase retained intracellularly was similar for wild-type, control, and  $chc^-$  cells. Also, as previously observed, the relative level of acid phosphatase enzyme activity in wild-type cells is almost threefold that of the glycosidases.

To more accurately quantify the secretion defect, standard secretion assays were performed. In these assays, logarithmically growing cells were harvested and resuspended in either growth media or starvation buffer, and lysosomal enzyme activity was measured in both cells and media at various times (Fig. 8). During growth conditions,  $\alpha$ -mannosidase enzyme was secreted from both wild-type and control cells at the same rate (11–12% of the total enzyme activity was found in the media at 30 min) (Fig. 8 A). By 4 h,  $\sim 45\%$  of the total  $\alpha$ -mannosidase enzyme activity was found outside of wild-type and control cells. In contrast, after 4 h,  $\sim 10\%$  of the enzyme activity was found in the extracellular media in  $chc^-$  cultures (Fig. 8 A). To determine whether the secretion defect was specific for  $\alpha$ -mannosidase, we examined the secretion of other lysosomal hydrolases. We found that the  $chc^-$  cells undersecreted another glycosidase,  $\beta$ -glucosidase, to the same extent as  $\alpha$ -mannosidase (data not shown). In addition, we examined a third enzyme, acid phosphatase, previously shown to be secreted during growth with slower



**Figure 7.** Intracellular retention of lysosomal enzymes in  $chc^-$  cells vs wild-type cells. Logarithmically growing cells were separated into cellular and media fractions by centrifugation, lysed with Triton X-100, and each fraction was assayed for lysosomal enzyme activity. In  $chc^-$  cells (black bars), 45–50% of the glycosidases,  $\alpha$ -mannosidase, and  $\beta$ -glucosidase were cell-associated compared to only 10–15% of the enzymes in the wild-type (white bars) and control cells (hatched bars). Acid phosphatase was cell-associated to the same extent in all cell types tested.





**Figure 8.** *Chc<sup>-</sup>* cells secrete mature lysosomal enzymes to a lesser extent than wild-type or control cells. The media was removed from growing cells and replaced with either media (growth conditions) or 10 mM phosphate buffer (starvation conditions). Samples were removed at the times indicated, the cells were pelleted and solubilized, and both the cell samples and supernatants were assayed for lysosomal enzyme activity. The results are expressed as the percent of the total enzyme activity (cell + media) that is extracellular at each time point. (A and B) The percent of extracellular enzyme activity of  $\alpha$ -mannosidase and acid phosphatase during growth for *chc<sup>-</sup>* (●), control (□) and Ax2 (Δ) cells. (C and D) The percent of extracellular enzyme activity of the lysosomal enzymes under starvation conditions for *chc<sup>-</sup>* (●), control (□), and Ax2 (Δ) cells. *Chc<sup>-</sup>* cells secreted mature lysosomal  $\alpha$ -mannosidase to a lesser extent than did the wild-type or control cells. During starvation, all cell types tested were able to respond to developmental cues and induce the secretion of mature lysosomal enzymes compared to the levels of enzyme that are secreted during growth.

kinetics than  $\alpha$ -mannosidase (Cardelli, 1993; Bush et al., 1990). We confirmed that the secretion rate and extracellular accumulation level of acid phosphatase was significantly less than that of  $\alpha$ -mannosidase in control cells. Interestingly, the kinetics with which *chc<sup>-</sup>* cells secreted acid phosphatase were almost identical to wild-type cells (Fig. 8 B), suggesting that the secretion defect was specific for a subset of the lysosomal hydrolases.

In *Dictyostelium*, limiting nutrient conditions initiate the developmental cycle. An early response to starvation is the induced secretion of lysosomal hydrolases (Cardelli, 1993).

To test this response, standard secretion assays were performed as described above using cultures of cells resuspended in starvation buffer. After 30 min of starvation, Ax2 and control cultures had relative levels of extracellular  $\alpha$ -mannosidase that were four times the levels observed during growth (Fig. 8, A vs C). By 4 h of starvation (secretion was linear for 1 h), ~60–75% of the total  $\alpha$ -mannosidase enzyme activity in Ax2 and control cells was found in the extracellular fraction, 1.5–2 times the level observed for these cultures in growth media (compare A to C). Under starvation conditions, *chc<sup>-</sup>* cells consistently secreted less of  $\alpha$ -mannosidase than did control or Ax2 cultures. After 1 h in starvation buffer, *chc<sup>-</sup>* cells had secreted 25% of this enzyme in the media, whereas control cells had secreted 60% in the extracellular media (Fig. 8 C). By 4 h in starvation buffer, *chc<sup>-</sup>* had secreted 52% of  $\alpha$ -mannosidase compared with 66–77% of the enzymes secreted by control and wild-type cells. Although *chc<sup>-</sup>* cells secreted less  $\alpha$ -mannosidase than control cells, the levels secreted during starvation conditions were significantly greater than the levels achieved under growth conditions. After 1 h in starvation buffer, the relative level of extracellular  $\alpha$ -mannosidase was 10 times the levels attained during growth (Fig. 8, A vs C). By 4 h, the extracellular fraction reached 52% of the total  $\alpha$ -mannosidase activity, five times the levels attained during growth conditions. Thus *chc<sup>-</sup>* cells remained responsive to the developmental cues that increase secretion rates of the hydrolases. Fig. 8 D indicates that both the rate and the extent of secreted acid phosphatase increased in cultures of starved *chc<sup>-</sup>* cells. Further, the increase in secretion was similar for wild-type, control and *chc<sup>-</sup>* cultures; after 4 h in starvation buffer, the three cultures secreted ~30–40% of their acid phosphatase compared to the 10–12% of the enzyme that was secreted after 4 h in growing cultures.

## Discussion

We have investigated the role of the clathrin heavy chain in membrane traffic by analyzing the properties of *Dictyostelium* cells engineered to eliminate a functional clathrin heavy chain gene. The cells are viable and provide us with an experimental model to study the transport of markers and proteins in a cell that is devoid of clathrin. Our investigation of the mutant cells identifies roles for the clathrin heavy chain in (a) the internalization of fluid from the plasma membrane; (b) sorting of precursor hydrolases from the constitutive secretory pathway to the lysosomal pathway; and (c) secretion of mature  $\alpha$ -mannosidase from the lysosome to the extracellular space.

### The Role of Clathrin Heavy Chain in Endocytosis

We find that clathrin-minus cells exhibit a severe defect in the internalization of fluid from the extracellular media. Relative to the parental axenic strain or control transformants, *chc<sup>-</sup>* cells internalize fluid-phase markers at only one fourth the rate and extent of wild-type cells. These results confirm previous studies of clathrin-minus *Dictyostelium* generated by antisense plasmids (O'Halloran and Anderson, 1992b). The *chc<sup>-</sup>* mutant described here demonstrates that clathrin is not essential for viability in *Dictyostelium* cells. In addition, we can now conclude that there is a clathrin-independent endocytosis pathway that constitutes a minor compo-



ment of total endocytosis in *Dictyostelium*. Previous studies have shown that endocytosed markers can recycle to the cell surface from a postlysosomal compartment (Padh, 1993). Our results show that this recycling pathway is not absolutely dependent on clathrin.

### ***The Role of the Clathrin Heavy Chain in Protein Sorting to the Lysosome***

Previous studies have shown that the clathrin heavy chain plays a role in protein sorting from the secretory pathway. In the yeast *Saccharomyces cerevisiae*, a mutant strain carrying a temperature-sensitive allele of the clathrin heavy chain exhibited a severe but transient protein sorting defect at the nonpermissive temperature, suggesting that the clathrin heavy chain was involved in protein sorting (Seeger and Payne, 1992a). The transient nature of the defect suggests that a clathrin-independent sorting mechanism may have compensated for the loss of clathrin heavy chain function. In *Dictyostelium*, we have found that the absence of clathrin heavy chain caused a significant decrease in the efficiency of lysosomal protein sorting; ~25% (representing a 5–10-fold increase over wild-type) of newly synthesized  $\alpha$ -mannosidase precursor molecules were missorted and rapidly secreted in the unprocessed form via the default constitutive secretory pathway. As in the yeast system, there appears to be a clathrin heavy chain-independent mechanism of protein sorting in *Dictyostelium* since ~75% of radiolabeled  $\alpha$ -mannosidase precursors were correctly sorted to the lysosome.

It is thought that the initial cleavage of  $\alpha$ -mannosidase that generates the 80- and 58-kD subunits from the 140-kD precursor occurs in the late Golgi or in an endosomal compartment, an event that may be necessary for sorting to lysosomes (Wood and Kaplan, 1985; Richardson et al., 1988). A delay in reaching this cleavage compartment could result in the rapid secretion of the uncleaved precursor through the default secretory pathway. The clathrin heavy chain specifically affects the sorting of proteins before the cleavage compartment since only the sorting of the 140-kD precursor was affected in *chc*<sup>-</sup> cells. The efficient processing of the 80-kD intermediate form suggests that once  $\alpha$ -mannosidase is in or through the cleavage compartment, it is correctly localized to dense lysosomes. One model that can explain these results is that the clathrin heavy chain segregates newly synthesized precursor molecules from the constitutive secretory pathway by selectively packaging the precursor molecules into vesicles destined for the cleavage compartment. This scenario is analogous to a mechanism proposed for lysosomal enzyme targeting in mammalian cells, where lysosomal enzymes bound to MPRs are packaged into clathrin-coated vesicles destined for the lysosomal pathway. However, the absence of MPRs in *Dictyostelium* suggests either that the lysosomal precursors segregate in clathrin coated vesicles or that a novel receptor mediates this interaction. In addition, the *Dictyostelium* lysosomal enzymes are not completely missorted in the absence of the clathrin heavy chain, suggesting the existence of a clathrin-independent mechanism of lysosomal protein sorting to the cleavage compartment or the activation in *chc*<sup>-</sup> cells of a compensatory targeting pathway.

The missorting and oversecretion of  $\alpha$ -mannosidase precursor molecules in *chc*<sup>-</sup> cells could also be explained by

an alternative model in which the protease that cleaves the newly synthesized precursors into the intermediate forms is localized into the cleavage compartment by a clathrin-mediated pathway. In the absence of clathrin, the protease would not be localized properly and the precursor would not be processed properly. The result of decreased processing would be reflected as the oversecretion of the precursor form. This possibility is consistent with studies that have shown that inhibition of the proteolytic processing of precursors for  $\alpha$ -mannosidase and  $\beta$ -glucosidase cause the oversecretion of precursor forms of these enzymes (Richardson et al., 1988). This alternative hypothesis is supported by studies in yeast that show that some proteases require clathrin to localize to a late Golgi compartment (Seeger and Payne, 1992b; Payne and Schekman, 1989).

### ***The Role of Clathrin in Secretion of Mature Lysosomal Enzymes***

There are at least two pathways by which lysosomal enzymes can be exported from the cell: either the enzymes are missorted as uncleaved precursors and secreted through the default constitutive secretory pathway, or the lysosomally localized mature enzymes are exported through lysosomes through a regulated pathway. The clathrin heavy chain does not affect the transport or export through the constitutive secretory pathway because the uncleaved  $\alpha$ -mannosidase precursor was efficiently secreted from *chc*<sup>-</sup> cells. However, our results indicate that the clathrin heavy chain is directly or indirectly involved in the secretion of mature enzymes from lysosomes. We find that both the rate and extent of secretion of the lysosomal enzyme  $\alpha$ -mannosidase was significantly reduced in *chc*<sup>-</sup> cells. This effect was specific for  $\alpha$ -mannosidase: the absence of clathrin did not affect the secretion of another hydrolase, acid phosphatase. Differences in secretion behavior of acid phosphatase and  $\alpha$ -mannosidase have been found in previous studies that show that the mature lysosomal glycosidases are secreted from *Dictyostelium* cells at a different rate and with different kinetics than acid phosphatase (Cardelli, 1993). The mechanism of differential secretion between lysosomal enzymes has been postulated to be due to a preferential intracellular retention of acid phosphatase over the glycosidases; perhaps by a tighter association of acid phosphatase with an intraorganelar matrix and/or an increase in the efficiency of packaging of other enzymes into secretory transport vesicles. Thus, the clathrin heavy chain may act to increase the efficiency of selective packaging of  $\alpha$ -mannosidase into the transport/secretory vesicles destined for the extracellular milieu and without clathrin,  $\alpha$ -mannosidase is inefficiently secreted at rates similar to acid phosphatase. Our results show that *chc*<sup>-</sup> cells remain able to respond to starvation signals and induce the secretion of the mature lysosomal enzymes. The three- to fivefold increase in the level of both  $\alpha$ -mannosidase and acid phosphatase enzyme secretion exhibited by *chc*<sup>-</sup> cells demonstrates that the developmental induction of mature lysosomal release is not blocked in the absence of the clathrin heavy chain.

It is worth speculating where the clathrin heavy chain may function in the clathrin-dependent secretory pathway. Secretion of mature lysosomal enzymes is thought to progress in at least two steps: first, from the lysosome to an intermediate

compartment, termed the postlysosomal compartment, and from this compartment to the plasma membrane for release into the extracellular environment (Padh et al., 1993). Vesicles filled with enzyme cargo may bud directly from the lysosome and transport the enzyme to the intermediate compartment. This may be a step analogous to endocytosis in that clathrin heavy chain may be required for the budding process from the lysosome. Alternatively, the lysosomal organelle may mature into the postlysosomal compartment, and the clathrin heavy chain may facilitate this process. The second step of the secretory pathway is the movement from the postlysosomal compartment to the plasma membrane. Again, this process may occur either by budding of transport vesicles from the postlysosomal compartment or by the maturation and fusion of this compartment to the plasma membrane. The clathrin heavy chain may function at either of these two stages of the clathrin-dependent pathway. Studies are currently underway to determine the site at which the clathrin heavy chain affects secretion of mature lysosomal enzymes.

We are grateful to Dr. John M. Bush for Northern analysis of the cDNAs. We also thank the members of the Cardelli and O'Halloran laboratories for critical reading of this manuscript and for helpful scientific discussions.

This research is supported by National Institutes of Health grant DK 39232-05 (J. Cardelli and National Science Foundation grant DCB-9104576 (J. Cardelli) and National Institutes of Health grant GM48625 (T. O'Halloran). T. O'Halloran is the recipient of a Basil O'Connor Starter Scholar Research Award. We also acknowledge the support of the Louisiana State University Medical Center Center for Excellence in Cancer Research and the Center for Excellence in Arthritis and Rheumatology.

Received for publication 15 March 1994 and in revised form 19 April 1994.

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