Dictyostelium discoideum Mutants with Temperature-sensitive Defects in Endocytosis

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Abstract. We have isolated and characterized temperature-sensitive endocytosis mutants in *Dictyostelium discoideum*. *Dictyostelium* is an attractive model for genetic studies of endocytosis because of its high rates of endocytosis, its reliance on endocytosis for nutrient uptake, and tractable molecular genetics. Endocytosis-defective mutants were isolated by a fluorescence-activated cell sorting (FACS™) as cells unable to take up a fluorescent marker. One temperature-sensitive mutant (indy1) was characterized in detail and found to exhibit a complete block in fluid phase endocytosis at the restrictive temperature, but normal rates of endocytosis at the permissive temperature. Likewise, a potential cell surface receptor that was rapidly internalized in wild-type cells and indy1 cells at the permissive temperature was poorly internalized in indy1 under restrictive conditions.

Growth was also completely arrested at the restrictive temperature. The endocytosis block was rapidly induced upon shift to the restrictive temperature and reversed upon return to normal conditions. Inhibition of endocytosis was also specific, as other membrane-trafficking events such as phagocytosis, secretion of lysosomal enzymes, and contractile vacuole function were unaffected at the restrictive temperature. Because recycling and transport to late endocytic compartments were not affected, the site of the defect's action is probably at an early step in the endocytic pathway. Additionally, indy1 cells were unable to proceed through the normal development program at the restrictive temperature. Given the tight functional and growth phenotypes, the indy1 mutant provides an opportunity to isolate genes responsible for endocytosis in *Dictyostelium* by complementation cloning.

ENDOCYTOSIS plays an important role in a wide variety of functions in eukaryotic cells, including nutrient uptake, internalization of receptors, antigen processing, and retrieval of membrane inserted into the cell surface during secretion. In animal cells, endocytosis typically involves the budding of clathrin-coated pits from the plasma membrane to yield coated vesicles that rapidly uncoat and fuse with early endosomes, a heterogeneous population of tubules and vesicles scattered throughout the cytoplasm. In early endosomes, internalized plasma membrane components and extracellular macromolecules are sorted such that proteins to be recycled back to the cell surface are separated from those destined for transport to late endosomes and lysosomes. A great deal is known about the kinetics and morphology of the intracellular compartments involved in mammalian cells, but the molecular mechanisms for these various steps remain poorly understood.

A number of proteins that play important roles during endocytosis have been identified. These include low molecular weight GTPases of the rab and ARF families, members of which have been implicated in regulating the formation or fusion of endosomes (Chavrier et al., 1990; Lenhard et al., 1992; Serafini et al., 1991; van der Sluijs et al., 1991). At least in vitro, endosome fusion may also involve NSF and one or more annexins (Diaz et al., 1989; Gruenberg and Emans, 1993). Finally, clathrin, clathrin-associated adaptors, and dynamin are all essential components in the formation of coated vesicles from the plasma membrane (Kosaka and Ikeda, 1983; Lin et al., 1991; Pearse and Robinson, 1990; van der Bliek et al., 1993). Ordering the steps in which these molecules function, identifying further components of functional significance, or assessing their relative importance in intact cells, has proved difficult.

In contrast to our understanding of endocytosis, a wealth of information concerning the mechanisms and organization of the secretory pathway has emerged from exploitation of *Saccharomyces cerevisiae* as a genetic model (Novick et al., 1980). While this approach has the potential to greatly increase our understanding of endocytosis, current genetic strategies for studying endocytosis in yeast have thus far met with relatively limited success (Chvatchko et al., 1986; Raths et al., 1993). While yeast cells share at least some features in common with endocytosis in animal cells (Raymond et al., 1992; Vida et al., 1993; Wichmann et al., 1992), progress has been hampered by yeast's low rates of internalization and by the presence of a cell wall that limits the access

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of endocytic markers to the cells. In addition, clathrin is of limited importance, while genetic evidence suggests a role for actin in yeast endocytosis, unlike mammalian cells (Kubler and Riezman, 1993). The marker receptors used for most yeast endocytosis studies, α-factor receptor and a-factor receptor, are not typical of classical coated pit receptors but more similar to the mammalian β-adrenergic receptor, a polytypic signaling receptor not generally associated with clathrin-coated pits (Dohlman et al., 1991). Although endocytosis and the tools for studying the endocytic pathway are highly developed in animal cells, the generation of mammalian mutants of endocytosis has mostly yielded cell lines with defects in organelle acidification (Merion et al., 1983; Roff et al., 1986; Schmid et al., 1989; Timchak et al., 1986). Moreover, mammalian cells suffer from a lack of tractable somatic and molecular genetics.

Because of these difficulties, we sought to develop a genetic model to study endocytosis using the simple eukaryote *Dictyostelium discoideum*. Like yeast cells, *Dictyostelium* cells have a small haploid genome that is highly amenable to mutant selection and molecular genetic manipulations such as homologous recombination (De Lozanne and Spudich, 1987; Harloff et al., 1989; Loomis, 1969). Like animal cells, they lack a cell wall and can mediate both phagocytosis of large particles and pinocytosis of extracellular fluid. Indeed, *Dictyostelium* cells exhibit a capacity for endocytosis that is comparable to mammalian macrophages, a cell type that exhibits among the highest rates of endocytosis known (Steinman et al., 1976; Thilo and Vogel, 1980). Moreover, *Dictyostelium* cells also appear to depend on endocytosis as their primary means for obtaining nutrients.

Many features of the endocytic pathway in *Dictyostelium* cells are closely related to the endocytic pathway in animal cells. Clathrin-coated pits have been observed (Swanson et al., 1981), and cells lacking the clathrin heavy chain gene are unable to internalize fluid phase markers (O'Halloran and Anderson, 1992). After internalization, extracellular macromolecules pass through endosomal and lysosomal compartments of decreasing pH (reaching a pH of ~5) (Padh et al., 1993). Internalized material eventually reaches a neutral pH compartment that eventually fuses with the plasma membrane, expelling nondigestible contents into the extracellular space. Nevertheless, like animal cells, *Dictyostelium* cells exhibit rapid recycling of plasma membrane components (Thilo and Vogel, 1980), as well as the intracellular sorting and transport of lysosomal enzymes (Cardelli et al., 1989; Ebert et al., 1989a; Freeze et al., 1989; Richardson et al., 1988).

The biological necessity of endocytosis for the survival of *Dictyostelium* cells provides a good opportunity to design genetic screens for the isolation of mutants defective in this process. Since it is possible to isolate wild-type copies of mutant genes by complementation cloning, *Dictyostelium* would appear to be an attractive model for the genetic analysis of endocytosis in eukaryotes. In this paper, we report on the isolation and characterization of initial temperature-sensitive endocytosis mutants.

Materials and Methods

Cells

D. discoideum AX4 cells were used as the parental cell line for mutagenesis

and were provided by David Knecht (University of Connecticut, Storrs, CT). Cells were grown in HL5 media + 0.1 mg/ml ampicillin + 0.1 mg/ml streptomycin in 100-mm plastic tissue culture dishes or shaken at 150–180 rpm at 21°C unless otherwise noted. Cell count was monitored using an hemacytometer.

Chemicals

FITC-dextran (Sigma Immunochemicals, St. Louis, MO). (70,000 mol wt was dissolved in 20 mM K/NaPO$_4$ buffer, pH 6.3, and extensively dialyzed or filtered over a G25 Sepharose PD-10 column (Pharmacia Biotech, Piscataway, NJ) to remove free FITC and stored as frozen aliquots. N-methyl, N'-nitro-N-nitrosoguanidine and p-nitro- phenylmanno-pyranoside were purchased from Sigma Immunochemicals. Luciferin and luciferase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Endocytosis Assay

Cells were washed, spun at 200 g for 4 min, and resuspended to a concentration of 4 × 10^6 cells/ml in HL5 media. FITC-dextran was added to a final concentration of 2 mg/ml and cell suspensions were swirled at 150–180 rpm. 1-ml aliquots were stopped in 6 ml ice-cold 20 mM K/NaPO$_4$ buffer, pH 6.3. Samples were subsequently washed in 6 ml 20 mM K/NaPO$_4$ buffer, pH 6.3, and then in 6 ml 10 mM glycine, pH 9.3. Cell pellets were lysed in 2.5 ml 50 mM glycine, pH 9.3, 0.3% Triton X-100 for 10–30 min on ice, and cell debris was removed by a 1,200 g spin for 10 min. Fluorescence of the lysates was measured in a fluorescence spectrofluorometer (LS-5; Perkin-Elmer Corp., Norwalk, CT). Excitation and emission wavelengths were 485 and 515 nm, respectively. A standard curve for FITC-dextran was linear throughout the range of fluorescence intensities measured.

Mutagenesis

AX4 cells were grown shaking culture to a density of 8 × 10^5 cells/ml. 2 × 10^8 cells were washed in and resuspended to a final concentration of 6.6 × 10^6 cells/ml in sterile 20 mM Na/KPO$_4$, pH 6.3, buffer. N-methyl, N'nitro-N-nitrosoguanidine dissolved in DMSO was added to a final concentration of 1 mg/ml, and the cells were incubated for 30 min at 18°C with swirling. The cell suspension was added to 20 ml cold HL5 and spun at 200 g for 5 min. The cells were washed 2× with 40 ml cold HL5, resuspended in 50 g/liter heat-killed *Klebsiella aerogenes*, and separated into four flasks and grown at 18°C. Viability after mutagenesis was assessed from viability plates set up for each mutagenesis condition and a nonmutagenized control by spreading triplicate SM plates with 200 μl *K. aerogenes* and 100 cells/plate. SM plates were made as described in Sussman (1987) with the following modifications: 1.0 g yeast extract, 0.43 g MgSO$_4$, and 0.6 g K$_2$PO$_4$. Killing rates after mutagenesis were 85%.

Mutant Screen

Cells were allowed to recover after mutagenesis by growth at 18°C in a suspension of 50 g/liter heat-killed *K. aerogenes*. 2 d before sorting, cells were washed free of bacteria by repeated differential centrifugation (three times at 200 g for 5 min), and were resuspended in HL5 growth media. After 4 h at 18°C, the cultures were transferred to 26.5°C for 18 h. Before uptake, cells were resuspended in fresh media at a concentration of 2 × 10^6 cells/ml. Cells were incubated with 2 mg/ml FITC-dextran for 1 h and washed, as for the endocytosis assay. Cells were resuspended in 20 mM K/NaPO$_4$ buffer, pH 6.3, + 0.1 mg/ml ampicillin + 0.1 mg/ml streptomycin; + 20 μg/ml Trimethoprin/Sulfamoxole. Cells were sorted with a flow cytometer/sorter (FACSIV™, Becton Dickinson Immunocytometry Systems, San Jose, CA). The cells were excited at 488 nm, and the emission was collected through a 530/30 band pass filter. 1 × 10^7 cells were sorted in the first sort, 1 × 10^6 cells in the second sort, and 5 × 10^6 cells in the third sort. The 5–10% least fluorescent cells were collected by electrostatic separation into a suspension of 50 g/liter heat-killed *K. aerogenes*.

FITC Labeling of Salmonella typhimurium

Strain RE595 was grown overnight to saturation. 50 ml of the bacterial culture was pelleted and resuspended in 20 ml of 50 mM NaPO$_4$ buffer, pH 9.2. This was incubated with 0.1 mg/ml fluorescein isothiocyanate at 21°C for 3 h at 200 rpm. The bacteria were washed with PBS + Ca^{++}, Mg^{++} until the supernatant was colorless.

Biotinylation Assay

Cells cultures were divided in half, diluted to ~1 × 10⁶ cells/ml, and incubated for 2 h at 21°C or 26.5°C. Cells were collected, washed once in cold 20 mM K/NaPO₄ buffer, pH 8.0, and resuspended to 1 × 10⁷ cells/ml in the same buffer. A sample of unbiotinylated cells was taken. N-hydroxysuccinimidyl disulfide–biotin (Pierce Chemical Co., Rockford, IL) was added to a final concentration of 1 mg/ml. Cells were incubated 20 min on ice with occasional gentle shaking. Biotinylation was stopped by adding 10 ml cold quenching buffer. Quenching buffer is 50 mM NH₄Cl, 1 mM MgCl₂, 1 mM CaCl₂, and 20 mM K/NaPO₄ buffer, pH 6.3. Cells were spun and washed once with 10 ml cold quenching buffer. Cells were resuspended to 2 × 10⁶ cells/ml in HL5 + 5 mg/ml BSA (media was prewarmed to 21°C or 26.5°C) and incubated in shaking suspension. At the indicated times, 0.5-ml duplicate samples were transferred to tubes on ice. Samples to be stripped were spun and resuspended in stripping buffer and incubated for 10–15 min on ice. The stripping buffer consisted of 50 mM 2-mercaptoethanesulfonic acid, 75 mM NaCl, and 1 mM EDTA, pH 8.5. This incubation was repeated. Samples were washed twice in 1.4 ml cold 20 mM K/NaPO₄ buffer, pH 6.3. After addition of nonreducing sample buffer, samples were immediately boiled for 3 min. Samples were run on 7.5% SDS-PAGE gels and transferred to nitrocellulose. The nitrocellulose was blocked for 30 min in 5% nonfat dried milk, 0.5% Triton X-100 in PBS, and incubated for 30 min with 1 μg/ml HRP-streptavidin in the same solution. The blot was washed repeatedly in 20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween-20. Chemiluminescence was carried out following the manufacturer's instructions for enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Cellular ATP Measurement

Cells were shifted to 21°C or 26.5°C at 1 × 10⁶ cells/ml in 20 mM Na/KPO₄, pH 6.3. The cells were incubated in buffer during the temperature shifts to eliminate any differences in cellular energy levels caused by differences in nutrient internalization. After 1 and 2 h, 1 × 10⁶ cells were removed and lysed on ice in 100 μl 2% TCA for 30 min. The samples were neutralized by addition of 590 μl 130 mM Tris-acetate, pH 7.75. A luciferin/luciferase reagent was made by mixing 8 μl of 1 mg/ml luciferase with 5 ml of 0.2 mg/ml luciferin in 2× ATP assay buffer. 2× ATP assay buffer is 40 mM Hepes, 20 mM MgCl₂, 4 mM EDTA, and 0.36 mM DTT. The luciferin/luciferase reagent was kept in the dark and cold. An equal volume of luciferin/luciferase reagent was added to the diluted sample in the dark. Immediately after addition of the luciferin/luciferase reagent, the samples were placed in a scintillation counter and counted for 0.1 min. The square root of the counts were calculated and compared to an ATP standard curve.

α-Mannosidase Assay

Cells were incubated in 20 mM Na/KPO₄, pH 6.3, at a concentration of 5 × 10⁶ cells/ml at 21°C or 26.5°C. At 0, 1, and 2 h, 2 × 10⁶ cells were removed. The media and the cell pellet were separated and lysed at 0°C in 0.1% Triton X-100 plus protease inhibitor cocktail of 10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin at 0°C. The 0.37-ml samples were incubated with 20 μl of 100 mM NaOAc, pH 5.0, and 4 μl of 500 mM p-nitro-phenylmanno-pyranoside for 30 min at 35°C. The color reaction was stopped by addition of 0.4 ml 1 M Na₂CO₃. Samples were spun at 10,000 rpm for 5 min in an Eppendorf centrifuge, and the absorbance was measured at 405 nm.

Degradation Assay

Cells were incubated at 2 × 10⁷ cells/ml with 10 mg/ml FITC-BSA in HL5 at 21°C for 5 min in shaking suspension. The incubation was stopped with ice-cold HL5 + 5 mg/ml BSA and followed by two washes in the same media. The cells were resuspended to 5 × 10⁶ cells/ml in fresh, prewarmed media, and were incubated at 21°C or 26.5°C. 2-ml samples were collected at 0, 60, 90, and 120 min. The samples were spun, and the supernatant was divided in half. Half of the supernatant was precipitated in 15% TCA solution on ice for 30 min. The TCA soluble supernatant was collected. The cell pellets were solubilized in 1 ml 0.5 M glycine + 0.3% Triton X-100. Cell pellet, supernatant, supernatant TCA-soluble samples, and standards were adjusted to the same concentration of TCA, glycine, and media. The pH was adjusted to 8.5. Fluorescence of the samples was measured in a Perkin-Elmer LS-5 fluorescence spectrofluorometer. Excitation and emission wavelengths were 485 and 515 nm, respectively. The percent of degraded BSA = (TCA soluble in the media-0 min TCA soluble supernatant)/(total cpm) × 100%.

Contractile Vacuole Labeling

Cells were plated onto 22 × 22-mm coverslips. After attachment, cells were shifted to 26.5°C for 6 h. Coverslips were placed in a viewing chamber for the confocal microscope and 1 μg/ml FM 4-64 in 20 mM K/NaPO₄ buffer, pH 6.3, was added. Cells were imaged at 488 nm using the fluorescein isothiocyanate excitation filter block on a confocal scanning laser microscope (MRC 600; Bio Rad Labs, Microscience Division, Cambridge, MA) equipped with an argon/krypton laser (Bio-Rad Labs). Images were collected every 15 s on an optical memory disk recorder (model TQ-3031F; Panasonic Industrial Co., Secaucus, NJ).

Development Assay

Cells were grown in suspension with 50 mg/ml heat-killed K. aerogenes to a density of 8 × 10⁶ cells/ml. Cells were washed twice with ice-cold 20 mM K/NaPO₄ buffer, pH 6.3, and once with development buffer (55 mM NaPO₄, 20 mM KCl, and 2.5 mM MgCl₂) to remove bacteria. Cells were resuspended in development buffer to 4 × 10⁷ cells/ml. 0.1 ml cell suspension was spread on 60-mm development plates (development buffer + 2% agar). Plates were incubated at 21°C or 26.5°C in light for a day. Photos were taken with a camera attached to a dissecting scope (Wild Leitz MPS 52 M10; E. Leitz, Inc., Rockleigh, NJ).

Results

Isolation of Temperature-sensitive Endocytosis Mutants

To isolate mutants with temperature conditional defects in endocytosis, cells were first selected for defects in the internalization of a marker of fluid phase endocytosis. Cells of the axenic Dictyostelium strain AX4 (hereafter referred to as "wild type") were mutagenized with N-methyl,N'nitro, nitrosoguanidine to 15% survival rate, and were allowed to recover for 4 d at 21°C. They were shifted to the restrictive temperature (26.5°C) for 18 h to allow expression of the mutant phenotype, and then incubated in medium containing FITC-dextran for 1 h at 26.5°C. The cells were analyzed with a fluorescence activated cell sorter (FACS)® and the 5–10% least fluorescent cells were collected and allowed to recover for 3 d before repeating the screen. After three rounds of enrichment, cells were diluted out to clonal growth and were visually screened for FITC-dextran endocytosis. Fig. 1 shows a FACS® profile of the third sort of a mutagenized cell population and shows enrichment of a population of faintly fluorescent cells. However, a majority of cells retained wild-type levels of fluorescence. This probably reflected the normal heterogeneity of internalization within the wild-type population leading to selection of normal cells that then may enjoy some growth advantage over mutant cells. For example, wild-type mitotic cells are likely to not be endocytic, and would be repeatedly collected as internalization deficient. Nevertheless, a population of cells was retrieved that stably exhibited an inability to internalize FITC-dextran.

Characterization of Endocytosis Mutants

A number of temperature-sensitive (ts)[1] and nonconditional mutants were isolated exhibiting a variety of endocytosis and growth phenotypes (Table I). Some clones had nearly normal rates of endocytosis at the permissive temperature, and they exhibited marked reductions after 2 or 5 h at 26.5°C. Other clones exhibited partial defects at the permissive temperature and even greater defects at the restrictive temperature. Still, other clones showed no conditional defect, with endocytosis

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1. *Abbreviation used in this paper*: ts, temperature-sensitive.
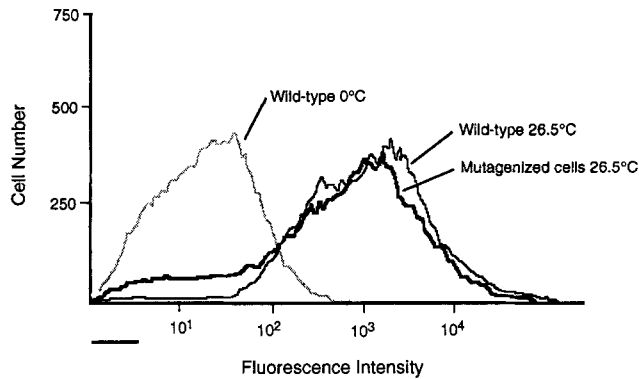
Figure 1. FACS® profile of the third sort of mutagenized cells after FITC-dextran internalization. Mutagenized cells were shifted to the restrictive temperature for 18 h, followed by a 55-min incubation with 2 mg/ml FITC-dextran. Wild-type cells were incubated with FITC-dextran at 26.5°C and 0°C for controls. The bar represents the 5% dimmest fluorescent cells that were collected from this sort.

being partially reduced at 21°C and 26.5°C. Several cell lines were also monitored for growth. In general, those with the strictest ts endocytosis phenotype also displayed the strictest ts growth phenotype (Table I).
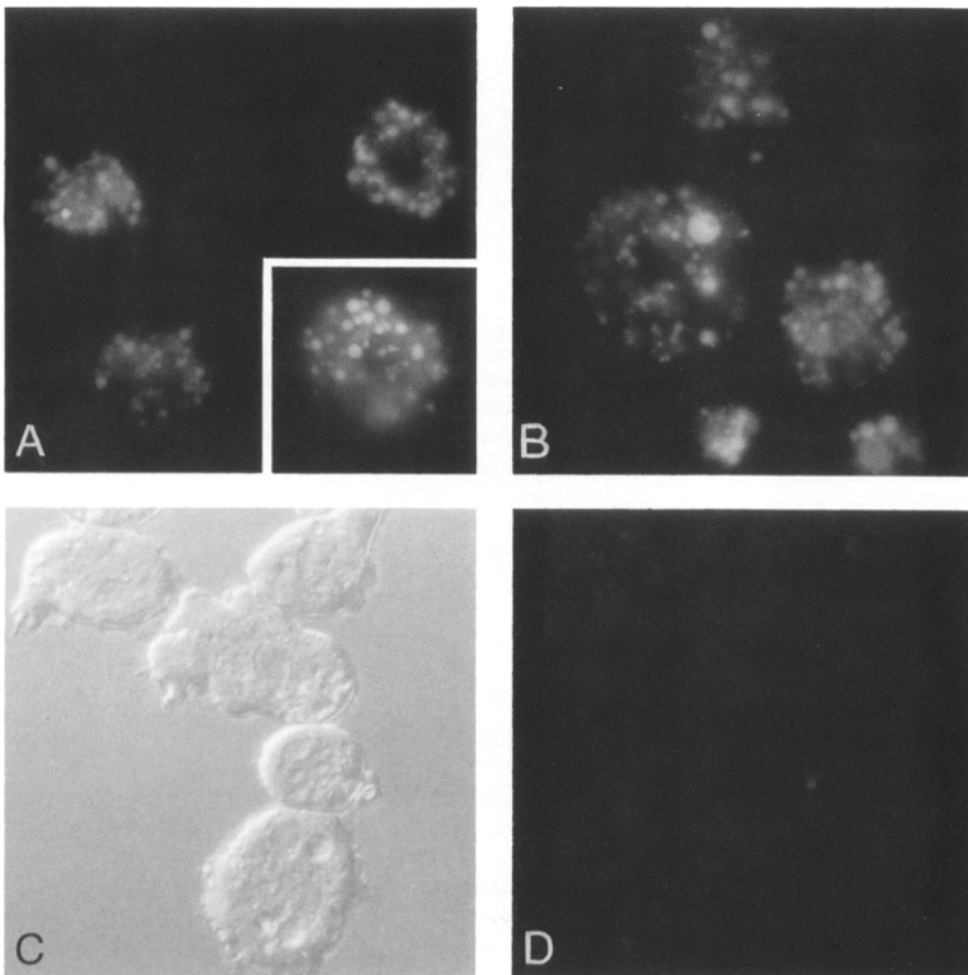
Mutant indy1 Has a Temperature-sensitive Endocytosis and Growth Defect

We selected one clone with the most severe ts defect for

Table I. Endocytosis and Growth Phenotypes of Dictyostelium Mutants

Strain	Internalization of FITC-dextran 21°C	26.5°C 2 h	5 h	Growth at 26.5°C (doubling time)
AX4 (Wild-type)	100%	100%	100%	12 h
HM40 (indy1)	97		8	NG
HM35	83		11	NG
HM36	88	50	22	NG
HM34	91	59	61	
HM22	70	54		40
HM28	54	41		36
HM24	34	11		
HM26	41	19		
HM29	40	42		
HM32	42	34		
HM23	29	27		
HM31	27	29		
HM33	39	43		
HM25	18	15		
HM30	79		64	
HM27		59		

Cells were incubated at the permissive (21°C) or restrictive (26.5°C) temperature for time indicated in axenic growth media. An internalization assay was initiated by addition of 2 mg/ml dialyzed FITC-dextran. After 1 h, the cells were washed, lysed, and the cell-associated fluorescence was quantified. AX4 cells internalized 5.63 ± 0.59 $\mu l/4 \times 10^6$ cells/h $\pm$ SE. Growth in axenic media in shaking culture was monitored at 21°C and 26.5°C. *NG*, indicates no growth.

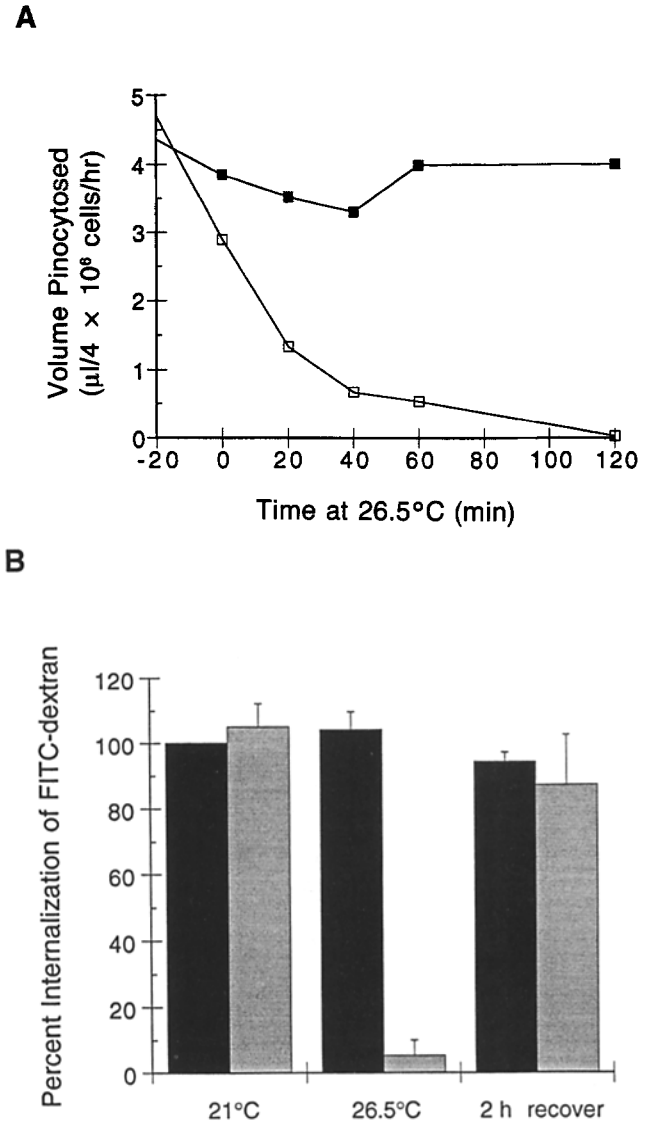*Figure 2.* indy1 demonstrates a temperature-sensitive endocytosis defect. (*A*) Cells were preincubated at 21°C or 26.5°C for 1 h in axenic media, and then incubated with 2 mg/ml FITC-dextran for 20 min. After washing, live cells were photographed on a fluorescence microscope using a fluorescein filter. (*A*) Wild type at 26.5°C; inset shows a further example of internalization by wild-type cells. (*B*) indy1 at 21°C. (*C*) Phase micrograph of the field shown in *D*. (*D*) indy1 at 26.5°C.

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A

B
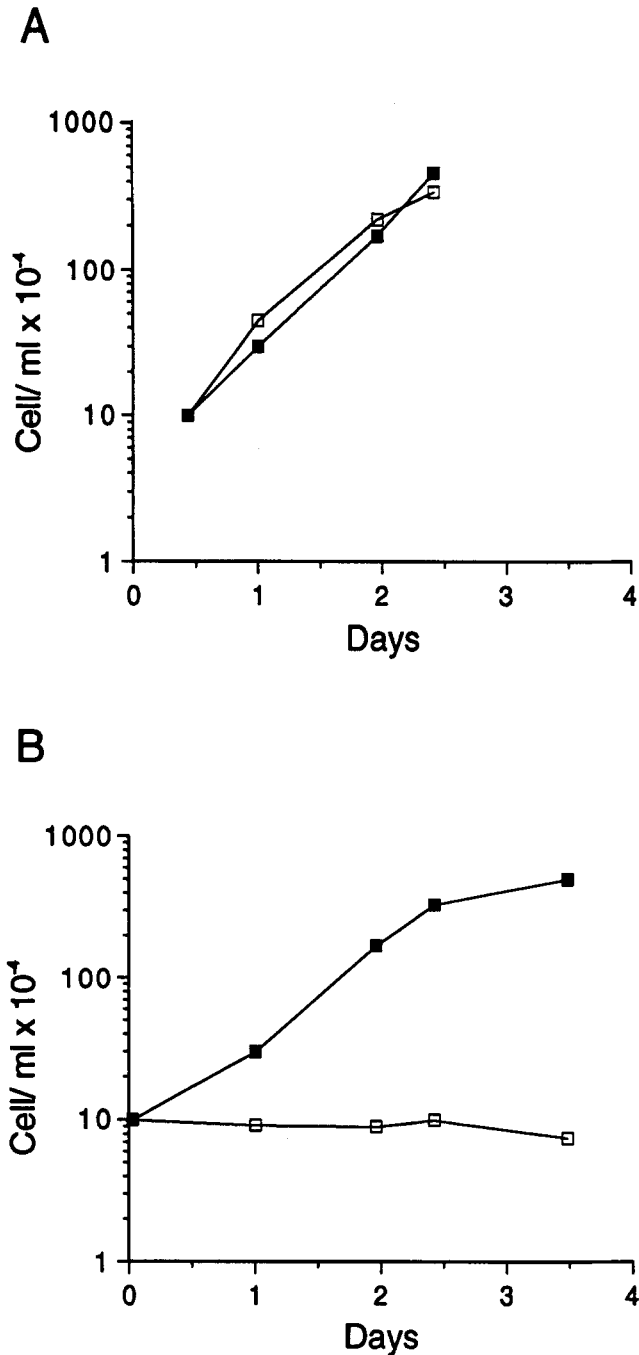
Figure 3. indy1 displays temperature sensitive growth. Cells were grown axenically in shaking culture at 21°C (*A*) or 26.5°C (*B*). Cell growth was measured by cell counting with a hemocytometer. —■—, wild type; —□—, indy1.

A

Time at 26.5°C (min)

B

Figure 4. The endocytosis defect of indy1 is rapidly induced and reversible. (*A*) Cells were incubated in axenic growth media at 26.5°C for the times indicated. Cells were incubated with FITC-dextran for 20 min, and an internalization assay was performed. The −20-min time point represents cells assayed at 21°C. —■—, wild type; —□—, indy1. (*B*) indy1 and wild-type cells were preincubated at 26.5°C for 2 h in axenic growth media. A 20-min internalization assay was performed at 26.5°C on one half of the cells, and the other half was returned to 21°C for 2 h before assaying for endocytosis at the permissive temperature. An assay was also performed on cells at 21°C. Error bars represent standard error from three experiments. ■, wild type; ▨, indy1.

detailed analysis. Clone HM40, renamed indy1 (for internalization dysfunction), was completely blocked in endocytosis at the restrictive temperature of 26.5°C. Fig. 2 shows fluorescence microscopy images of indy1 cells that were preincubated at the restrictive temperature for 1 h and then incubated with FITC-dextran for 20 min. Both wild-type and indy1 cells at the permissive temperature demonstrated a vesicular fluorescence staining. However, at the restrictive

temperature, almost no fluorescence could be detected in indy1 cells.

Indy1 cells also exhibited a tight ts growth defect. There was no growth at all in liquid media or on bacteria at 26.5°C, while there was normal growth under both conditions at the permissive temperature (Fig. 3). Growth in liquid media was restored by shifting back to 21°C even after 12 h at nonpermissive conditions, demonstrating the reversibility of the growth phenotype (not shown).

The Endocytic Defect in indy1 Cells Is Rapid and Reversible

The indy1 mutant showed a rapid and dramatic loss of ability to internalize FITC-dextran. As shown in Fig. 4 *A*, a significant decrease in FITC-dextran uptake was evident immediately upon shifting to the restrictive temperature. Relative to cells assayed at 21°C, indy1 cells incubated at 26.5°C for just the 20-min uptake assay period already exhibited a 40% decrease in FITC-dextran accumulation. Endocytosis continued to decrease rapidly thereafter and was virtually undetectable when a 120-min preincubation at the restrictive temperature preceded the 20-min uptake assay. Thus, indy1 cells exhibited a complete loss of endocytotic capability after only short times at the restrictive temperature. (For comparison, the generation time for *Dictyostelium* at 26.5°C in axenic media is ~10 h). Endocytosis in parental (wild-type) cells was not affected by the temperature shift (Fig. 4 *A*). Similar results were obtained if HRP was used instead of FITC-dextran (not shown). Using HRP as a tracer, we also examined wild-type and mutant cells by electron microscopy after diaminobenzidine cytochemistry to label endocytic organelles. Although there was a reduction in the number of structures labeled in indy1 cells incubated at the restrictive temperature, there was no obvious difference in the morphology of vesicles found in wild-type or mutant cells at either temperature (not shown).

Although complete, the endocytosis defect (like the growth defect) was reversible. After a 2-h incubation at the restrictive temperature, 87% of control FITC-dextran uptake activity was restored within 2 h after the cells were returned to 21°C (Fig. 4 *B*) indicating that incubation at the restrictive temperature did not block endocytosis due to a loss of cell viability. Nor was incubation at the restrictive temperature found to affect cellular ATP levels, an indicator of metabolic health (Table II).

Endocytosis of a Cell Surface Protein Is Defective in indy1 Cells

We next sought to determine whether the mutant was defective in receptor-mediated endocytosis. Since appropriate cell surface receptors have not yet been identified in *Dictyostelium*, we took a more general approach and compared internalization of labeled plasma membrane proteins in wild-type and mutant cells. Plasma membrane proteins were labeled by incubating cells with an amino-reactive, reducible biotin analogue at 0°C. After warming to 21°C for various periods of time, the cells were again placed on ice. The biotin label was removed from any biotinylated proteins still present on the cell surface by reduction of the disulfide bond with a cell-impermeant reducing agent. Thus, biotinylated protein that was intracellular was protected from removal by reduction, and the amount of labeled protein remaining gave a measure of endocytosis. This assay was successful in demonstrating rapid internalization of at least one plasma membrane protein in wild-type cells. Fig. 5 shows a time course of protection of plasma membrane proteins in wild-type cells under permissive conditions. A number of prominent bands, including bands at ~45, 50, 60, 70, 100, 120, and >200 kD, are labeled with biotin, but only the band at ~120 kD was protected after the shift to 21°C. The large band at 70 kD is present in unlabeled cells (data not shown) and is probably
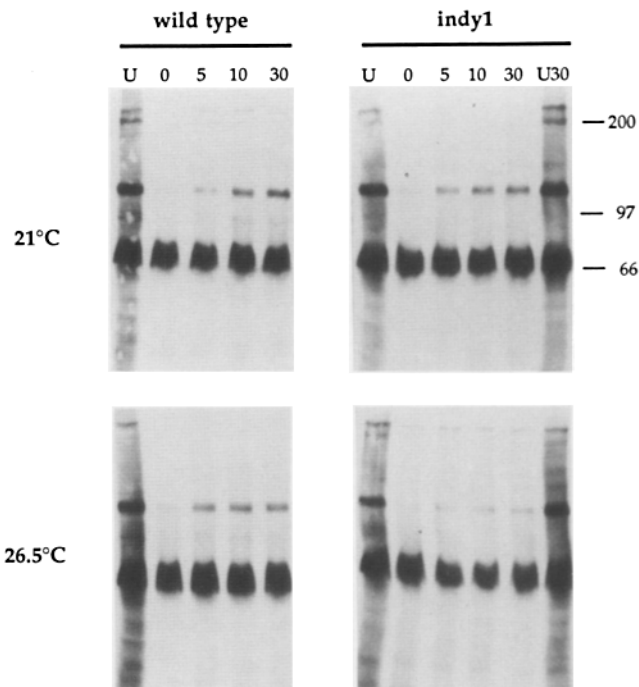
Figure 5. indy1 exhibits a defect in internalization of a plasma membrane protein. Cells were preincubated at 21°C or 26.5°C for 2 h, cell surface proteins were labeled with a cleavable biotin analogue at 0°C, and cells were warmed again to 21°C or 26.5°C to allow internalization of the labeled proteins for 0, 5, 10, and 30 min. At the indicated times, cells were cooled, and the cell surface biotin label was stripped. Internalized proteins were separated by SDS-PAGE, transferred to nitrocellulose, blotted with streptavidin-HRP, and visualized by chemiluminescence. The band present in all lanes at 70 kD is an endogenous biotin-containing protein. The lanes labeled U are unstripped samples at 0 min chase, and they demonstrate that a number of plasma membrane proteins can be labeled with the biotin moiety. The numbers indicate time of chase in minutes. Lane *0* demonstrates that the majority of the label is removed from the surface in samples stripped after a 0-min incubation after labeling. Lane *U30* indicates an unstripped sample at 30 min chase.

an endogenous biotin-containing enzyme, such as a carboxylase (Lydan and O'Day, 1991). Within 5 min, the 120-kD protein became protected, and by 10 min, the amount of label protected was stable, suggesting that an equilibrium between internalization and recycling was reached. (At that time, ~20% of the labeled protein was internal). The protein was also protected within the same time course at 26.5°C in wild-type cells. Although its identity is unknown, the 120-kD protein provides a useful marker as a rapidly internalized plasma membrane protein.

In indy1 cells at the permissive temperature, internalization of the 120-kD protein continued at the same rate and extent as wild-type cells, but there was very little protection of the 120-kD protein at 26.5°C (Fig. 5). This corresponds to what we observed with fluid-phase markers of endocytosis in indy1. The lack of protection of the protein in the indy1 cells at 26.5°C was not caused by loss of the biotin label or 120-kD protein from the cells during the chase, as indicated by the fact that the unstripped 30-min chase sample has as
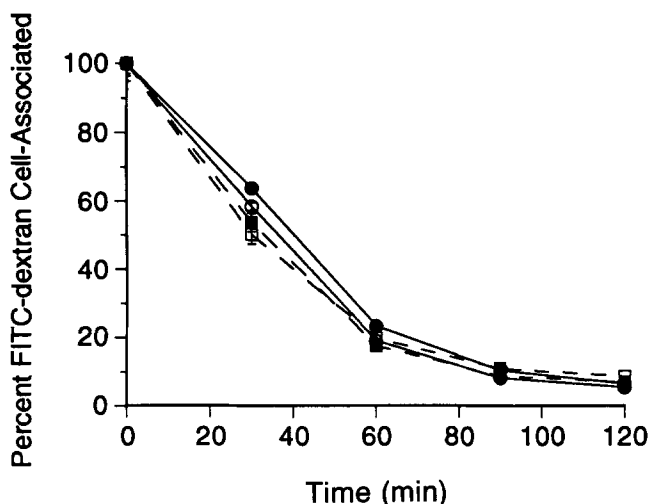
much labeling of the 120-kD protein as the unstripped 0-min chase sample (Fig. 5, lane *U30* vs. lane *U*). These data demonstrate that endocytosis of the 120-kD protein is temperature-sensitive in indy1, and it mimics the fluid-phase endocytosis defect of indy1.

Phagocytosis Is Unaffected in the indy1 Mutant

The defect was restricted to endocytosis, and did not include phagocytosis of large particles. As in mammalian leukocytes, phagocytosis in *Dictyostelium* is actin-dependent, and it involves the engulfment of large particles (≥ 1 μm in diameter). Phagocytic vacuoles then fused with lysosomes, resulting in the degradation of the internalized particle; in the case of *Dictyostelium*, this leads to the utilization released metabolites. To determine whether the defect in the indy1 mutant also affected phagocytosis, cells were incubated with FITC-labeled *K. aerogenes* or *Salmonella typhimurium* after preincubation at either the permissive or restrictive temperature. Ability to phagocytose bacteria remained at wild-type levels in the indy1 cells even after 4 h at the restrictive temperature (data not shown). This phenotype is in contrast to another mutant we have isolated (Cohen et al., 1994) which exhibits a phagocytosis defect but no deficiency in endocytosis. In addition, we have been able to "cross" indy1 cells with this mutant. The resulting cells were haploid but exhibited a normal phenotype with respect to endocytosis, phagocytosis, and ts growth. These data provide evidence that the endocytosis-defective indy1 cells are defective in a process genetically distinct from phagocytosis.

The indy1 Mutation Does Not Affect Recycling

Having established that the indy1 defect caused a rapid and reversible block in growth and endocytosis, we next turned to identifying its probable site of action. Since assays of fluid phase endocytosis measure both internalization and recy-
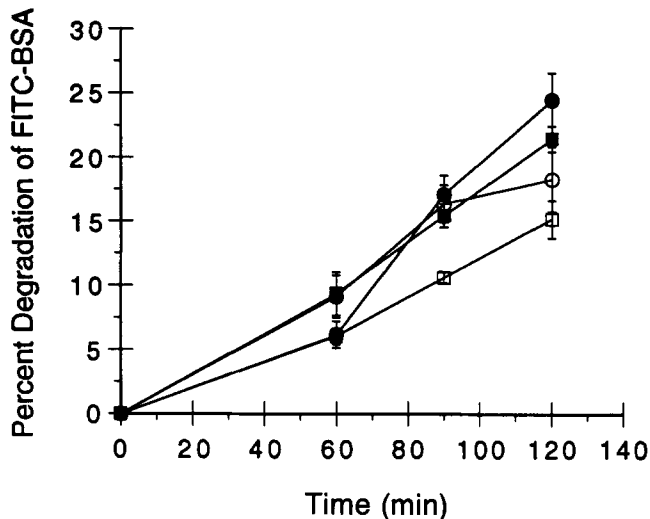
cling, we next designed an assay to selectively monitor recycling at the permissive and restrictive temperatures. Because of the rapid onset of the endocytosis defect, it was possible to load the cells under permissive conditions, shift then to 26.5°C, and determine the rate of FITC-dextran release back into the medium. As shown in Fig. 6, indy1 cells recycled FITC-dextran with kinetics indistinguishable from control cells at both 21°C and 26.5°C. After 2 h, >90% of the internalized FITC-dextran had been released into the medium. The released fluorescence was judged by gel filtration on Sephadex G25M to be intact FICT-dextran as opposed to free fluorescein (not shown). Thus, the inability of indy1 cells to accumulate FITC-dextran at 26.5°C was not caused by an increase in the rate of recycling.

Transport to the Lysosomes Is Not Impaired in indy1 Cells

It was also possible that the defect observed in indy1 reflected a primary block in the delivery of internalized material to lysosomes, a situation that might lead to a reduced capacity to accumulate endocytic tracers. This was tested by loading cells with FITC-BSA at 21°C for 5 min and then chasing them in the absence of the marker at 26.5°C for ≤ 2 h. It has been shown previously that at 5 min, the internalized material has not yet reached a hydrolase-containing compartment (Lenhard et al., 1992). After 0, 1, and 2 h, cells and media were collected, and the percentage of extracellular fluorescence that was not TCA precipitable was taken as the degree of degradation of the protein. No significant decrease in FITC-BSA degradation was observed in indy1 cells at 26.5°C when compared to either mutant cells at permissive condi-

Figure 6. Recycling is not affected in the indy1 mutant. indy1 and wild-type cells were incubated with 2 mg/ml FITC-dextran in axenic media for 1 h at 21°C. Cells were washed and shifted to fresh media at 21°C (*circles*) or 26.5°C (*squares*), and samples were collected at the indicated times. The fluorescence signal of both the cells and media was measured. (Solid symbols represent wild type; open symbols represent indy1.

Figure 7. Degradation of internalized BSA in not affected in indy1. FITC-BSA was internalized in wild-type and indy1 cells for 5 min at 21°C. Cells were washed and resuspended in fresh media and incubated at 21°C or 26.5°C for up to 2 h. Duplicate samples were taken at the indicated times, and were split in two. Half of the supernatant was TCA precipitated. The percent BSA degraded = (fluorescence in the TCA-soluble sample − fluorescence of 0 min supernatant)/total fluorescence of the cells and supernatant. The percent of degradation of FITC-BSA in cells kept at 0°C for 2 h was the same as in cells taken at 0 min of chase. ─■─, Wild type at 26.5°C; ─●─, wild type at 21°C; ─□─, indy1 at 26.5°C; ─○─, indy1 at 21°C.

tions or wild-type cells (Fig. 7). Since the internalization defect was manifested almost immediately after shifting indy1 cells to 26.5°C, but only a slight loss of degradation was observed after 2 h, it is unlikely that the primary defect of indy1 is a block in the delivery of endocytic tracers to lysosomes or other degradative compartments.
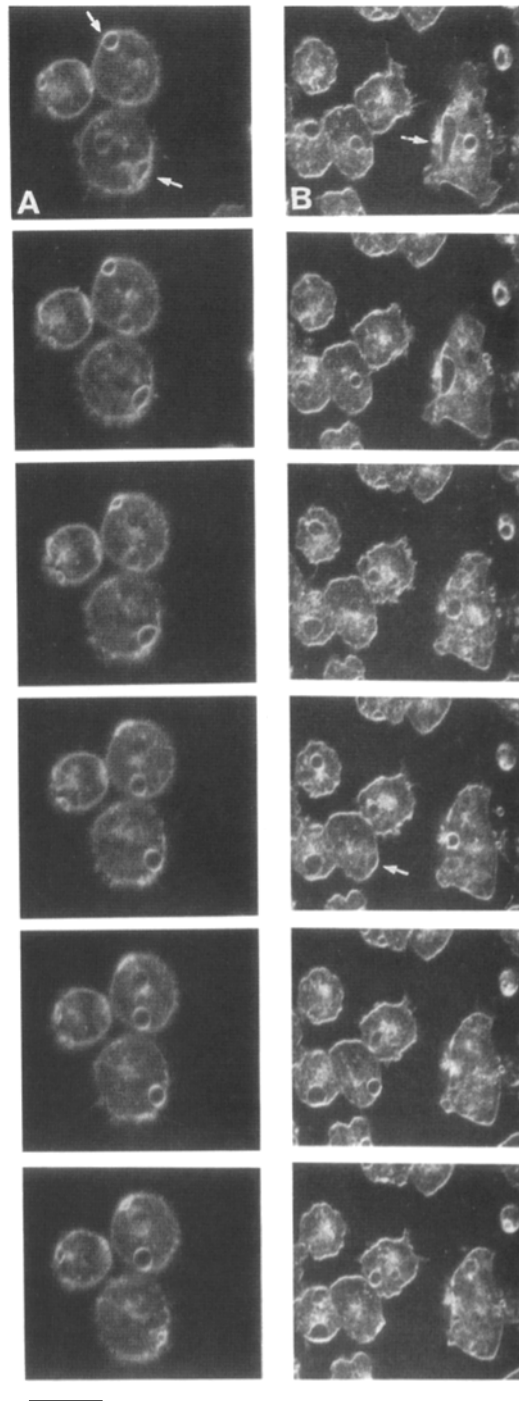
indy1 Cells Contain Functional Contractile Vacuoles

We next asked whether the indy1 mutations might affect contractile vacuole function, whose activity may be at least indirectly related to the endocytic pathway. The contractile vacuole in *Dictyostelium* (like other fungi) permits these cells to survive in low ionic strength environments by performing an osmoregulatory role in which these vacuoles undergo repeated rounds of filling with water and then fusing with the plasma membrane (Patterson, 1980). Clathrin-deficient cells completely lack contractile vacuoles, suggesting some role for the endocytic pathway in forming or maintaining the contractile vacuole (O'Halloran and Anderson, 1992). To test whether the indy1 mutant also lacked these vacuoles, we viewed live cells labeled with a styrene dye (FM4-64, obtained from J. Heuser, Washington University, St. Louis, MO) that preferentially labels the plasma membrane and contractile vacuoles on a confocal microscope. At both 21°C and 26.5°C, indy1 cells exhibited highly dynamic contractile vacuoles that enlarged and emptied in a fashion indistinguishable from wild-type cells (Fig. 8).

Secretion of α-Mannosidase Is Normal in the indy1 Mutant

Dictyostelium cells secrete newly and previously synthesized lysosomal enzymes continuously during vegetative growth and exhibit a stimulation of secretion upon entry into the developmental cycle (Dimond et al., 1981). Since *Dictyostelium* cells with defects in lysosomal targeting also show a partial reduction in endocytosis (Ebert et al., 1989*b*), and since the endocytic and secretory pathways may share some components in common, we next asked whether indy1 cells exhibited a ts defect in the secretion of α-mannosidase. Secretion was induced by transferring cells to starvation buffer to initiate development at either the permissive or restrictive temperatures. After 2 h at either temperature, wild-type cells were found to have released ~40% their total α-mannosidase activity into the media (Table II). indy1 cells secreted 35% and 37% of their total α-mannosidase activity at 21°C and 26.5°C, respectively. Although indy1 cells at the permissive temperature contained only half as much total α-mannosidase activity as wild-type cells, it was apparent that the indy1 mutation did not block secretion.

Taken together, the data suggest that the indy1 defect is specific to the endocytic pathway, and it is not a more general defect in cell health or membrane traffic because recycling, phagocytosis, and secretion occur and contractile vacuole function continues unabated. While we have not precisely identified the site of the indy1 block, it must affect an early stage(s) of the endocytic pathway since probes preloaded into cells continue to their final destinations even at the nonpermissive temperature. Furthermore, our data suggest that the indy1 protein acts in the clathrin-dependent pathway because both the indy1 and clathrin are not required in downregulation of cAMP receptor, but both are needed for internalization of a major plasma membrane protein that can be biotinylated (O'Halloran, T.J., personal communication).

Figure 8. indy1 cells contain functional contractile vacuoles. Wild-type (*A*) and indy1 cells (*B*) were shifted to 26.5°C for 6 h before labeling with 1 μg/ml FM4-64. Images were collected using a confocal scanning microscope. Images shown were taken 2 min apart. Arrows indicate contractile vacuoles filling with fluid and emptying at the plasma membrane. Bar, 7 μm.

indy1 Cells Exhibit a Reversible, Temperature-sensitive Defect in Development

Under starvation conditions, *Dictyostelium* cells enter a well-characterized developmental program. Briefly, the developmental cycle begins when cells respond to pulse of extracellular cAMP secreted by one cell. Cells migrate towards this chemotactic stimulus and begin emitting pulses of

Table II. ATP Levels and Secretion of α-Mannosidase Are Not Thermosensitive in indy1 Cells

| | ATP levels | | | Secretion of α-mannosidase | |
	21°C	26.5°C	Time	21°C	26.5°C
	nmol/10⁶ cell		*h*	*percent of enzyme activity in the media + SE*	
Wild type	2.9	3.1	1	27.4 ± 0.82	24.7 ± 3.92
			2	39.0 ± 0.40	37.5 ± 3.06
indy1	3.0	3.2	1	24.3 ± 1.29	27.0 ± 0.87
			2	35.3 ± 1.90	36.7 ± 2.37

Cellular ATP levels were measured in cells incubated at 21°C or 26.5°C in 20 mM Na/KPO₄, pH 6.3, for 1 h. The cells were incubated in buffer during the temperature shifts to eliminate any differences in cellular energy levels caused by differences in nutrient internalization. ATP levels were measured using a luciferin/luciferase assay. A representative experiment is shown.

Cells were shifted to 21°C or 26.5°C in starvation buffer. After 1 and 2 h, cells were removed from the media, and the cell pellets were assayed for α-mannosidase activity using a colorimetric assay. *SE*, standard error of three experiments.

cAMP, drawing still more cells into an aggregation center. About 100,000 cells stream into an aggregate of cells that undergoes a well-defined program of morphological changes that culminate in the formation of a mature fruiting body composed of a stalk supporting a sorus of spores. While the role of endocytosis in development is unknown, it is clear that patterns of protein secretion change as a function of development stage (Lenhard et al., 1989).

At the permissive temperature, the indy1 mutant exhibited a substantially normal developmental phenotype. As shown in Fig. 9, the mutant cells form mature, if somewhat shorter, fruiting bodies containing viable spores. The rate development was also slightly slower (30 vs 24 h in wild-type AX4 cells). If transferred to starvation conditions at the restrictive temperature, however, indy1 cells were unable even to stream together to form aggregates; instead, they remained as a homogenous lawn of cells (Fig. 9). O'Halloran and Anderson (1992) observed that cells rendered endocytosis-

defective by reduction in clathrin heavy chain expression also were defective in early development. Thus, there appears to be a requirement for endocytosis, or some other step(s) requiring clathrin and the indy1 gene, to initiate the development program.

As with the endocytosis defect, the development defect is reversible. After incubations of ≤18 h at the restrictive temperature under starvation conditions, indy1 cells on agar were subsequently able to complete development upon shift back to 21°C. Moreover, the mutation had the ability to arrest development at multiple stages during the cycle (Bacon, R., and data not shown).

Revertants Exhibit Partially Rescued Growth and Endocytosis Phenotypes

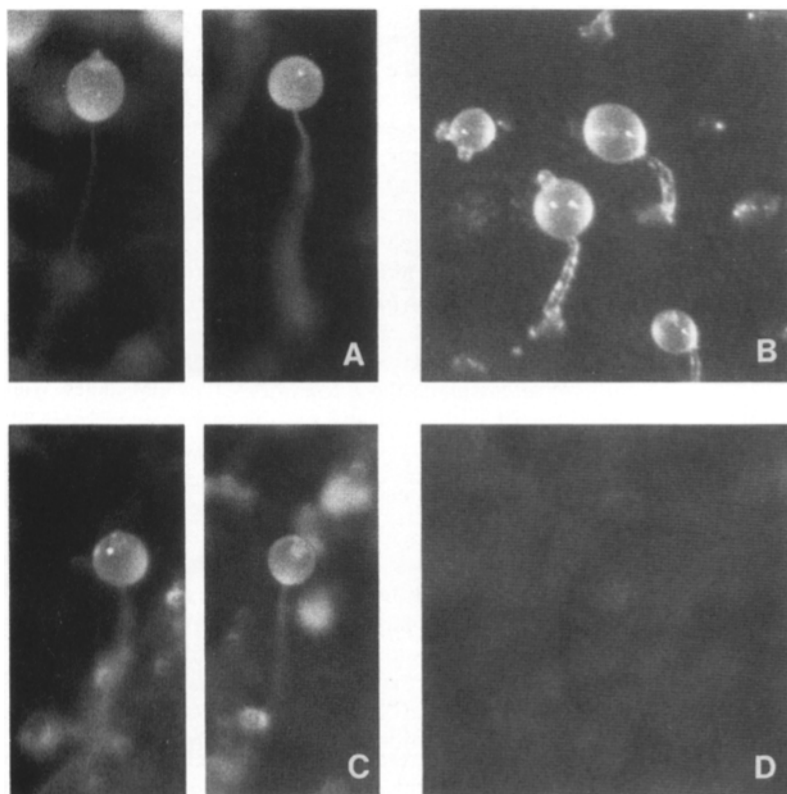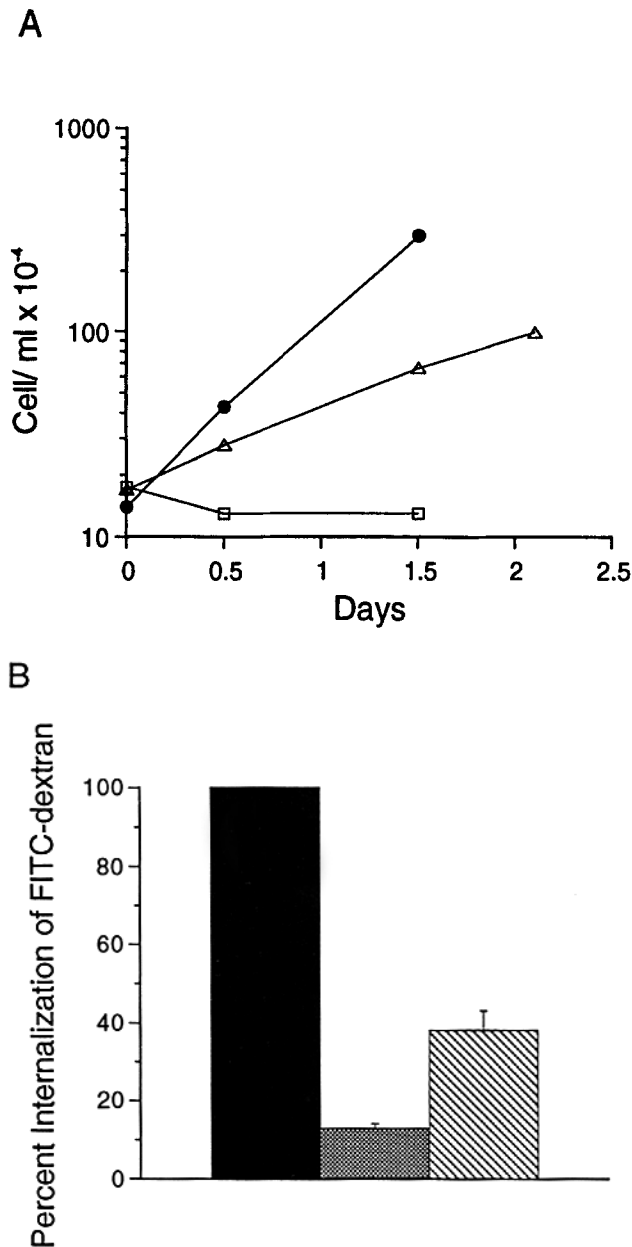To determine whether the phenotypes exhibited by indy1 cells were likely to be the result of single or multiple muta-

Figure 9. indy1 cells exhibit an *agg*-ts developmental phenotype. Cells were developed on development plates and photographed after 26 h. (*A*) Wild type at 21°C. (*B*) Wild type at 26.5°C. (*C*) indy1 at 21°C. (*D*) indy1 at 26.5°C.

A

B

Figure 10. A revertant of indyl has partially recovered growth and endocytosis phenotypes. (*A*) Cells were grown in axenic growth media at 26.5°C. Growth was measured by cell counting with a haemocytomer. -●-, Wild type; -□-, indyl; -△-, revertant. (*B*) Cells were shifted to 26.5°C for 1 h. A FITC-dextran internalization assay was carried out for 30 min. Cells were washed, lysed, and cell-associated fluorescence was quantified. Error bars represent standard error from three experiments. ■, Wild type; ▦, indyl; ▨, revertant.

tions, we sought revertants of indyl by selecting for growth at the restrictive temperature. Revertants were isolated at a frequency of 2 × 10⁻⁸. One such revertant was characterized in detail and found to have partially recovered its ability to grow in liquid media at 26.5°C. As shown in Fig. 10 *A*, this revertant exhibited a doubling time of 21 h, as compared to ~10 h for wild-type cells.

The indyl revertant also showed a partial rescue of the en-

docytosis phenotype. When FITC-dextran uptake was determined after a 1-h shift to 26.5°C, the revertant showed a threefold increase relative to indyl (Fig. 10 *B*). The partial rescue of both growth and endocytosis in the revertant suggests that the growth and endocytosis phenotypes of indyl are caused by a mutation at a single genetic locus. It is unlikely that the partially recovered endocytosis phenotype was simply a secondary result of the recovery of the ts growth phenotype because other mutants selected for ts growth defects (Clarke, 1978) do not demonstrate an endocytosis-defective phenotype (unpublished data).

The revertants also partially recovered the ability to grow on bacteria and to exhibit development under restrictive conditions. While neither development nor bacterial growth were rescued at 26.5°C, both phenotypes were normal at 24°C (data not shown). These data suggest that all of the phenotypes we observed in the indyl mutant are all linked to a single mutation that was partially, but coordinately, rescued in a single revertant.

Discussion

Our work has shown that *D. discoideum* will provide a new genetic approach for the study of endocytosis. A major advantage of this model relative to yeast is the commonalities in endocytosis between *Dictyostelium* and higher eukaryotes. *Dictyostelium*, like mammalian cells, have an extensive endocytic pathway that includes clathrin-coated pits and vesicles, acidic endosomes, phagocytic vacuoles, and lysosomes. Moreover, recent phylogenetic evidence suggests that a closer link exists between *Dictyostelium* and mammals than exists between yeast and mammals (Loomis and Smith, 1990). Of equal importance is the fact that conventional endocytosis assays are easy to perform using *Dictyostelium* cells because of the robust nature of their capacity for fluid phase uptake and the absence of a cell wall. Finally, the physiological relevance of endocytosis for *Dictyostelium* is indicated by its role in nutrient uptake.

On the other hand, the use of classical genetic techniques is limited in *Dictyostelium*. These cells lack a sexual cycle, and even parasexual genetics is complicated by the fact that diploid cell lines, in our hands, are difficult to maintain in stable form. Consequently, our attempts thus far to place our set of endocytosis mutants into genetic complementation groups have been largely unsuccessful.

We have isolated temperature-conditional endocytosis mutants from *Dictyostelium* and characterized one mutant, indyl, in detail. Indyl cells showed a dramatic loss of endocytosis at the restrictive temperature alone with a loss of ability to survive under these conditions. This defect affected endocytosis of both fluid and plasma membrane proteins. The thermoreversibility of the endocytosis phenotype, together with the fact that energy levels were not depleted in indyl under nonpermissive conditions, indicated that the loss of endocytosis was specific and not a secondary characteristic to a general loss of viability. The rapid induction of the phenotype and the fact that a variety of other cellular processes, including phagocytosis, secretion of the lysosomal enzyme α-mannosidase, and contractile vacuole function, were normal at the restrictive temperature also suggested that the defect in endocytosis was the primary phenotype of the mutation. Furthermore, the internalization

defect was probably not the result of a generalized defect in acidification of the endocytic pathway. A test for acidification was carried out on the mutant using the acidophilic dye acridine orange, which accumulates in acidic compartments. After preincubation at either the permissive or restrictive temperature, indy1 cells were able to accumulate acridine orange in vesicular structures like wild-type cells (data not shown), indicating that a major disruption in acidification mechanisms had not occurred. However, given the imprecise nature of this assay, we cannot rule out subtler effects on acidification in the mutant.

The ts growth defect of the mutant was closely coupled to the endocytosis defect. A revertant of indy1 demonstrated partial rescue of endocytosis, development, and growth at the restrictive temperature, providing genetic evidence that the defects in endocytosis, development, and growth are caused by a mutation in a single gene. The cells exhibited a ts growth defect on a bacterial food source and in liquid media. While this was not immediately expected given the phenotype of the indy1 mutant, there is a precedent for mutants in which the growth characteristics do not completely coincide with the phenotype of interest. Some yeast vacuolar protein sorting mutants are ts for growth, while the vacuolar protein sorting phenotype is expressed at both permissive and restrictive conditions (Raymond et al., 1992).

Temperature-sensitive mutants can be used to dissect the requirements of endocytosis and development in a manner not possible in nonconditional mutants. Accordingly, we were able to establish that the site of action of the indy1 defect is at a point early in the endocytic pathway. Preinternalized markers were either delivered to degradative compartments or recycled back to the cell surface normally, even at 26.5°C. Thus, the later stages of the endocytic pathway were not affected. Although electron microscopy did not reveal the accumulation of arrested coated pits or some other vesicular intermediate, these results suggest that the mutation may have resulted in a generalized cessation of endocytic vesicle formation.

The rapidity of onset of the defect suggests that either a long-lived protein becomes nonfunctional at the nonpermissive temperature, or that a rapidly turning-over protein is not properly folded when synthesized. Interestingly, it is known that rapidly turning-over protein(s) are required for continued endocytosis in Dictyostelium cells. Treatment of the cells with cyclohexamide to inhibit protein synthesis blocks both endocytosis and phagocytosis (Gonzalez and Satre, 1991; Bacon, R., unpublished data). Since phagocytosis was unaffected in indy1 cells, it is clear that the mutation did not reflect a generalized defect in protein synthesis.

Also, despite similarities in many phenotypes between the clathrin heavy chain deficient cells and indy1, it is unlikely that the clathrin heavy chain and INDY1 are the same genes because other phenotypic features in the mutants differ greatly. In contrast to clathrin-deficient cells, indy1 has normal contractile vacuole morphology and function. In addition, clathrin levels appears to be normal in indy1 cells (unpublished data).

One fascinating feature of endocytosis in Dictyostelium is the degree to which it is subject to regulation. The original D. discoideum isolate, NC4, can neither grow in liquid medium nor exhibit fluid phase endocytosis; as in the wild, they survive by phagocytosis of bacteria. Two unlinked

recessive mutations (axeA and axeB) allow for axenic growth and convert endocytosis to a process regulated by growth conditions (Kayman and Clarke, 1983; Williams et al., 1974); transfer from a bacterial food source to liquid medium induces endocytosis by >100-fold. How these two recessive mutations lead to a gain of function phenotype is unknown, but the AXEA and AXEB wild-type alleles must serve as negative regulators since NC4 cells are capable of synthesizing all of the structural components required for fluid phase endocytosis. In any event, it is conceivable that the indy1 mutation affects a gene product involved in the presumptive regulatory pathway controlled by these two genes.

One attractive possibility as to why Dictyostelium might regulate endocytosis so carefully is that endocytosis plays a critical function during the developmental cycle. Potential roles for endocytosis in the development cycle are readily recognized. Receptors or cell adhesion molecules on the cell surface may need to be internalized to downregulate receptor signaling or to allow for the morphogenic changes of development, respectively. Likewise, the balance of membrane may need to be maintained by endocytosis to allow for continued secretion of spore coat proteins and cellulose needed for morphogenesis. Furthermore, treatment of developing cells with NH4Cl disrupts development and promotes prespore formation (Weeks and Gross, 1991). Recent evidence suggests that this is accomplished by alkalinizing the endocytic pathway (Davies et al., 1993). Thus, it is of added significance that indy1 cells exhibit a development phenotype. At the restrictive temperature, they cannot even aggregate. It is also interesting that development can be arrested at multiple stages, depending on the time of the temperature shift (data not shown).

Supporting evidence from studies in other organisms lend credence to the notion that endocytosis may play a critical role in development. The Drosophila shibire[ts] mutant is a well-characterized endocytosis mutant that also exhibits a development phenotype. Shibire flies become reversibly paralyzed at the restrictive temperature because of a block in recycling of synaptic vesicle membrane from the neuromuscular junction (Koenig, 1989). Morphological analysis indicated that endocytosis is blocked at the point of coated pit formation so that coated pits are unable to pinch off from the plasma membrane (Kessell et al., 1989; Kosaka and Ikeda, 1983). Interestingly, the shibire protein has been found to be homologous to the mammalian protein dynamin, now know to play a role in coated vesicle formation (Herskovits et al., 1993; van der Bliek and Meyerowitz, 1991). In addition, shibire[ts] embryos exhibit a variety of developmental phenotypes, depending on the time of the temperature shift (Poodry, 1990; Poodry and Edgar, 1979; Swanson and Poodry, 1980, 1981). The phenotype of these mutants are suggestive of a role for a link between the cell autonomous function of endocytosis and the developmental fate of an organism.

Dictyostelium has proved amenable to molecular genetic manipulation. Given their high efficiency of genetic transformation, it should be possible to isolate novel genes from recessive mutants by expression cloning. For these reasons, Dictyostelium should prove to be extremely useful in the generation and characterization of novel genes required for the endocytic pathway.

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