A New Collection of Thermosensitive Endocytosis Mutants in the Cellular Slime Mold Dictyostelium discoideum

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ABSTRACT. We used a photoactivatable fluid-phase marker to isolate a new collection of thermosensitive endocytosis mutants in the cellular slime mold Dictyostelium discoideum. All the strains were thermosensitive for growth on bacteria or axenic medium at 27°C. Initial rates of endocytosis rapidly decreased upon incubation at the restrictive temperature, but surprisingly most of the strains showed a transient recovery of activity with prolonged exposure to 27°C. Endocytosis and exocytosis activities were uncoupled for some of the cell lines at 27°C whereas the others had to be shifted to 28°C. Further molecular analysis of these mutants could lead to the discovery of new proteins involved in endocytosis and its regulation.

Supplementary key words. Amoebae, genetics, pinocytosis.

Genetic screens have been used with great success to isolate mutants with defects in membrane traffic, especially those mutants that affect the secretory pathway [22]. There has been far less success defining the endocytic pathway. From biochemical studies, we know of the critical role of a number of proteins (clathrin, adaptors, rabs) but there clearly are many more proteins involved. Moreover, most of these studies concerned receptor-mediated endocytosis whereas fluid-phase-endocytosis (pinocytosis) is still poorly understood both at the mechanistic and regulatory levels. There have been a few previous genetic screens for endocytosis mutants. Notable successes were with mammalian cells [3, 18, 25], yeast [19, 22], slime molds [1, 2] and fruity flies [6, 9, 17]. Selective screening procedures using resistance to toxic substances entering only by endocytosis have primarily uncovered mutants in the regulation of acidification of endo-lysosomal compartments [2, 13, 14, 21].

The haploid genome of the vegetative Dictyostelium discoideum amoebae makes this organism very suitable for genetic analysis [12, 15]. As no amino acid or sugar transporters have been identified to date in the plasma membrane of Dictyostelium, pinocytosis is likely to be the only way for nutrient entry in axenically grown amoebae [4, 16]. Therefore Dictyostelium provides a good model for the study of this process. However, the total reliance on pinocytosis makes it necessary to screen for conditional mutants.

We previously described a new method to isolate thermosensitive endocytic mutants from Dictyostelium based on the ability of a photo-reactive probe generating singlet oxygen to kill the cells once taken up by pinocytosis [10]. We now report the successful application of this technique to isolate new Dictyostelium mutants with a defective endocytic pathway.

MATERIALS AND METHODS

Cell culture and growth measurements. Dictyostelium discoideum strain Ax-2 (American Type Culture Collection 24397) and thermosensitive mutants were grown axenically on shakers rotating at 170 rotations per minute (rpm) in a peptone-yeast extract medium containing maltose as a carbon source [26]. To measure growth rates, amoebae in their exponential phase of growth were diluted to 5 × 10^5 cells/ml in nutritive axenic medium either at 20°C or at 27°C. Cells were counted with a Coulter counter Model ZM at set time points on aliquot samples diluted in a solution containing 0.5% glutaraldehyde and five-fold diluted Isoton II from Coulter.

Isolation of thermosensitive endocytosis mutants. Exponentially growing Ax-2 amoebae (2 × 10^6 cells) suspended in 17.5 mM Na⁺,K⁺-Phosphate buffer pH 6.3 (Pi buffer) were treated for 30 min at 20°C with 0.5 mg/ml N'-methyl-N'-nitro-N-nitrosoguanidine [10]. After washing to get rid of the mutants, they were grown for 48 hours at 20°C to allow expression of the potential mutations. Cultures were then shifted for 16 hours to 27°C and then incubated with 10 μl/ml 4'3'3'-dioctodifluorescein isothiocyanate-dextran for four hours further in the dark. Extracellular photosensitizer was then removed by washing in ice-cold Pi buffer with 0.05% (w/v) bovine serum albumin (Pi-BSA buffer). Cells were illuminated at 4°C as described, transferred to nutritive axenic medium and grown for two to three days at 20°C in nonshaking conditions. Temperature-sensitive endocytic mutants survived this treatment, because they failed to take up the photoactivatable probe at 27°C. The selection procedure (27°C incubation—loading photosensitizer and photoactivation—growth at 20°C) was repeated at least five times before individual mutants were cloned. After each illumination cycle, survival rates were measured by plating on Klebsiella aerogenes lawns grown on the surface of a solid nutritive medium [23] and counting plaques formed after five days at 20°C. At the final stage, at least 100 colonies from each independent population were tested for their temperature sensitivity for growth (tsg) phenotype. Only one or two clones were selected among the numerous candidates, subcloned and stored as desiccated spores on silica gel.

Diploid selection. The ability of mutant strains to complement each other's defect was determined by the generation of diploids [12]. Each strain was cultured at 20°C on a K. aerogenes lawn (see above). Amoebae taken from the growing edge of the culture were transferred with a sterile loop (one loop per cross) to 5 ml Pi buffer. Cells were centrifuged at 1,200 g for 5 min and suspended in 250 μl 20 mM CaCl₂. Two strains were mixed (250 μl of each) in 24-multifwell plates and incubated overnight at 20°C on a shaking rotator (175 rpm). The mixture was then spread together with K. aerogenes in Petri dishes containing solid nutritive medium and put at 27°C. At that temperature, the bacteria grow faster than the amoebae. Plaques appearing in the bacteria lawn were diploid because complementation between the thermosensitive lines enables growth at 27°C. The boomerang shape and the larger size of the spores were used to confirm that the colonies were diploid [27].

Fluid-phase endocytosis assays. FITC-dextran (FD) was used as a fluid-phase marker [8]. Briefly, amoebae (5 × 10^6/ml) suspended in fresh culture medium were incubated in the presence of FD (1 or 2 mg/ml). To measure intracellular FD, aliquots (1 ml) of the cell suspension were taken as a function of time, washed twice in ice-cold Pi-BSA buffer suspended in 1 ml Pi buffer. After cell counting, amoebae were lysed with...
detergent at alkaline pH by addition of 2 ml 100 mM Na₂HPO₄, 0.25% Triton X-100 (v/v). The amount of FD was determined fluorometrically (excitation and emission wavelengths of 470 and 520 nm, respectively).

To follow the loss of endocytic activity at non-permissive temperature, Ax-2 or mutant strains (5 × 10⁶ cells/ml) were pre-incubated for variable periods at 27°C (or 20°C as a control) in axenic medium before adding 2 mg/ml FD. Samples taken at five-minute intervals were treated as described above. In the first 15 min, internalization rates were constant. Slopes corresponding to the initial rate of pinocytosis in each condition.

To study the effect of restrictive temperature on the maximum amount internalized, cells were loaded overnight with 1 mg/ml FD at 20°C at a final cell density of about 5 × 10⁶ cells/ml. They were then shifted to the non-permissive temperature, still in presence of FD in the intracellular medium, and 1 ml samples were treated as described above.

**RESULTS**

**Mutant isolation.** We adapted our photodynamic strategy [10] to isolate thermoconditional mutants defective in endocytosis. With this protocol, the selective killing of parental cells enriches for thermosensitive endocytosis mutants. We observed increased numbers of surviving cells after each round of photodynamic selection. In the experiment corresponding to the isolation of mutant HGR117 (see below), survival rates increased from less than 0.5% in mutagenized Ax-2 cells up to 18% after six photodynamic selection cycles (Fig. 1). The number of surviving amoebae varied from one experiment to the next, but there was always a marked enrichment through photodynamic killing. After each screen, 100 clones surviving the last illumination step were checked for the ∆sg phenotype by comparing growth on a lawn of the bacteria *Klebsiella aerogenes* at 20°C and at 27°C. Percentages of ∆sg clones after five illumination cycles varied from 2% to 50% with an average of 16%. Each ∆sg clone was subcloned and kept for long term storage as desiccated spores on silica gel beads at 4°C. We used this approach to isolate 26 clonal lines named HGR101 to HGR126.

**Diploid formation.** In a first stage, we investigated whether the selection procedure led to different mutants or if there was any bias towards a particular class of mutants. Fifteen mutant strains from our collection were crossed pairwise to test for complementation. The formation of a diploid growing at the non-permissive temperature demonstrated that the two strains that were fused had different temperature sensitive defects. Among a total of 105 possible combinations, we were able to produce 58 diploids that grew well at 27°C and nine crosses were negative (Fig. 2). These negative crosses were repeated at least once to confirm the result. The combined results showed that most of the strains such as HGR106, HGR110, HGR115, HGR121, HGR122, HGR124 and HGR126 have different mutations. From the available negative crosses, the group formed by the six following strains: HGR102, HGR103, HGR105, HGR116, HGR117, HGR119 could have mutations in the same protein. Similar reasoning applied to the other group consisting of the two strains HGR120 and HGR123. Thus, in the subset of 15 mutants considered here, our selection procedure has led to the targeting of at least nine different loci involved in the endocytic process.

**Growth characteristics.** To investigate the primary defects in the mutants, we tested whether the ∆sg mutant strains could grow in axenic medium at the non-permissive temperature. In liquid growth medium, nutrients are exclusively taken up by fluid-phase pinocytosis in *Dictyostelium* Ax-2 amoebae. A growth defect at 27°C may reflect an inhibition of pinocytosis activity. Detailed growth curves were conducted for 17 mutants. Growth characteristics at the permissive temperature (20°C) were found similar to that of the parent strain Ax-2, with a plateau at 1.4–2.7 × 10⁶ cells/ml and a generation time of 8–11 hours (Table 1). In most cases, growth stopped within the first hours after the shift at the restrictive temperature. Figure 3 shows growth curves measured at 20°C and 27°C for Ax-2 and HGR117 as a representative mutant. The two mutant strains HGR118 and HGR121 had a somewhat different behavior. After shifting to the non-permissive temperature, they grew nor-
Table 1. Growth parameters of *Dictyostelium discoideum* Ax-2 amoebae and various mutants at 20° C and 27° C.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Doubling time (hours)</th>
<th>Plateau (x 10^7/ml)</th>
<th>Doubling numbers at 27° C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ax-2</td>
<td>8.0 ± 0.4</td>
<td>2.5 ± 0.3</td>
<td>50</td>
</tr>
<tr>
<td>HGR101</td>
<td>8.0</td>
<td>1.5</td>
<td>≤1</td>
</tr>
<tr>
<td>HGR102</td>
<td>8.0</td>
<td>2.7</td>
<td>≤1</td>
</tr>
<tr>
<td>HGR103</td>
<td>9.5</td>
<td>1.8</td>
<td>≤1</td>
</tr>
<tr>
<td>HGR105</td>
<td>9.0</td>
<td>1.7</td>
<td>≤1</td>
</tr>
<tr>
<td>HGR106</td>
<td>11.0</td>
<td>1.4</td>
<td>≤1</td>
</tr>
<tr>
<td>HGR107</td>
<td>8.5</td>
<td>1.9</td>
<td>≤1</td>
</tr>
<tr>
<td>HGR108</td>
<td>8.0</td>
<td>2.1</td>
<td>≤1</td>
</tr>
<tr>
<td>HGR109</td>
<td>8.0</td>
<td>1.7</td>
<td>≤1</td>
</tr>
<tr>
<td>HGR110</td>
<td>8.0</td>
<td>2.7</td>
<td>≤1</td>
</tr>
<tr>
<td>HGR111</td>
<td>8.0-8.7</td>
<td>1.8-1.9</td>
<td>≤1</td>
</tr>
<tr>
<td>HGR112</td>
<td>10.5-9.6</td>
<td>1.8-1.8</td>
<td>3</td>
</tr>
<tr>
<td>HGR113</td>
<td>8.0</td>
<td>2.1</td>
<td>≤1</td>
</tr>
<tr>
<td>HGR114</td>
<td>11.0</td>
<td>1.7</td>
<td>≤1</td>
</tr>
<tr>
<td>HGR115</td>
<td>8.0</td>
<td>2.0</td>
<td>≤1</td>
</tr>
</tbody>
</table>

* Number of doublings observed after the shift at 27° C before growth arrest.

* Mean ± SD from four separate experiments.

Effect of the nonpermissive temperature on the initial rate of pinocytosis in the mutant strains. We used FD as a fluid-phase marker to directly measure the effect of the thermosensitive mutations on pinocytosis. This activity was characterized by two different parameters: the initial rate of fluid influx (R_{in}) and the plateau, which is reached when influx and efflux are in equilibrium [8]. The effect on R_{in} was considered first. At 20° C, R_{in} of each of our mutant strains (4.6 to 9.2 fl/cell/min, Table 2) was not very different from R_{in} of the parent line (8.0 fl/cell/min, Table 2).

We determined the effect of the restrictive temperature by measuring the initial rate of pinocytosis at different intervals after raising the temperature to 27° C. The parental strain Ax-2 was unaffected under these conditions. In HGR102 cells, R_{in} was strongly reduced within the first two hours at 27° C, decreasing from 5.6 to 1.0 fl/cell/min (Fig. 4). The uptake rate decreased even more at longer incubation times and was in fact undetectable after an overnight incubation at 27° C. The inhibition was completely reversible even after four hours at 27° C. In HGR102 cells, pinocytosis was rapidly restored up to the original level within three hours of incubation at 20° C (Fig. 4). Such continued decrease over time at 27° C was observed for

![Fig. 3. Axenic growth of *Dictyostelium discoideum* Ax-2, HGR117 and HGR118 amoebae at permissive and restrictive temperature. Amoebae from Ax-2 (panel 1), HGR117 (panel 2) and HGR118 (panel 3) strains were harvested from their shaking cultures at 20° C and suspended (5 x 10^6 cells/ml) at time = 0 in fresh nutritive medium at 20° C (C) or 27° C (O). Cell densities were then measured as a function of time.](image)

![Fig. 4. Reversible inhibition of fluid-phase pinocytosis at restrictive temperature in *Dictyostelium discoideum* HGR102. Amoebae were incubated at 5 x 10^6 cells/ml, either at 27° C (C) or at 20° C after a four-hour-preincubation at 27° C (O). At indicated times, initial rate of fluid entry was measured by adding FD (2 mg/ml) as a fluid-phase marker. Results are expressed as a percentage of control values at 20° C (see Table 2).](image)
two other mutant strains (HGR105 and HGR110, see Table 2). After four hours of incubation at 27°C followed by four hours at 20°C, full reversibility was observed for the mutant HGR105 and 75% of the original pinocytosis rate at 20°C was measured with HGR110.

The three strains that had a continuous loss of endocytosis at the restrictive temperature (HGR102, HGR105 and HGR110) were actually exceptions. All the other strains showed a transient recovery after a rapid loss of activity. Figure 5 shows HGR115 which was representative of this behavior. After 30 min at 27°C, fluid-phase endocytosis was reduced to 3.5% of the rate at 20°C. Remarkably, HGR115 cells recovered 50% of the activity after two more hours at 27°C. However, $R_{\text{recovery}}$ decreased to 5% of the control rate (20°C) following even longer incubations. This transient recovery did not occur at 29°C. Parental Ax-2 amoebae were unaffected at 27°C and still maintained 80% of their activity at 29°C.

Thus, we distinguish two groups among the sixteen mutants studied (Table 2). First, HGR102, HGR105 and HGR110 showed a continuous decrease of the initial rate of pinocytosis at restrictive temperatures. HGR102 was the most severely affected (20% of the control rate after two hours at 27°C). Second, HGR101, HGR103, HGR106, HGR115, HGR116, HGR117, HGR119, HGR120, HGR121, HGR122, HGR123, HGR124 and HGR126 lost 50 to 95% of their activity in the first hour at 27°C. These mutants transiently recovered after two hours reaching up to 80% of the control rates at 20°C, but then relapsed at longer incubation times with rates ranging from 5% to 50% after an overnight exposure at 27°C for the majority of the strains.

**Effect of nonpermissive temperatures on the plateau of pinocytosis.** To determine what effect the mutations have on the dynamic equilibrium between influx and efflux, amoebae were loaded with FD at 20°C up to plateau capacity, then shifted to the restrictive temperature. Intracellular FD was measured at different intervals at 27°C. The results are summarized in Table 3. At 20°C, the plateaus of the tsg mutants were very similar to those of parental Ax-2 amoebae. The plateau values corresponded to an apparent volume approximately equal to the cell volume (around 500 fl). Such high levels of endocytosed marker might reflect efficient concentrating steps along the endocytic pathway [24].

Figure 6 shows the detailed results of the experiment with HGR115 and HGR119 strains. In HGR115 cells, up to 40% of the fluorescent fluid-phase marker was expelled in the first two hours at 27°C, reflecting an arrest of influx while efflux still

![Figure 5](image)

**Fig. 5.** Effect of temperature on fluid-phase pinocytosis activity of *Dictyostelium discoideum* Ax-2 and HGR115 amoebae. Ax-2 (○, △) or HGR115 (□, △) amoebae (5 × 10⁶ cells/ml) were incubated at 27°C (□, △) or 29°C (○, △) for indicated periods before measuring the initial rate of pinocytosis. Results are expressed as a percentage of the control activities at 20°C (see Table 2).

![Figure 6](image)

**Fig. 6.** Effect of restrictive temperature on pinocytosis plateau of *Dictyostelium discoideum* HGR115 and HGR119 mutants. HGR115 (panel A) and HGR119 (panel B) amoebae were loaded at 20°C to their pinocytosis plateau by preincubating 5 × 10⁶ cells/ml for three hours with 1 mg/ml FD. At time 0, half of the amoebae suspension was shifted to 27°C (□) whereas the other half was maintained at 20°C (○). FD remained present in the Extracellular medium during the whole experiment. Intracellular FD was measured in each case as a function of time and expressed as a percentage of the value measured at time zero (see Table 3).
occurred. This arrest was followed by a transient increase of intracellular FD, similar to the observed recovery of the influx rate (Fig. 5). During this transient recovery, exocytic activity of HGR115 cells was stopped (not shown). In control experiments, we verified that the cells remained intact and showed no major changes in ATP levels over a period of seven hours at 27°C (not shown). In HGR119, the plateau was not significantly changed at the restrictive temperature, even though the R_{in} was reduced to 25%. This suggests that in this case, the exocytic process was also blocked.

Our mutant collection falls into two groups based on the plateau phenotypes. The first group of mutants lost intracellular FD at 27°C, suggesting that endocytosis was blocked but that exocytosis still occurred. This "leaking" phenotype was found for eight strains: HGR101, HGR102, HGR105, HGR106, HGR110, HGR115, HGR116 and HGR122. Among them, the decrease of the plateau was continuous for three strains: HGR102, HGR105 and HGR110. After an initial rapid loss, the five other strains showed a transient recovery of the plateau value (not shown). Thus, the situation was similar to the above described evolution of the R_{in} upon shifting at 27°C. HGR106 was the only strain to reach a new stable plateau when incubated at the restrictive temperature. This plateau was not dynamic but rather corresponded to a total arrest of fluid exchange between the inside and the outside of the amoebae (not shown).

A second group of five mutant strains: HGR103, HGR119, HGR120, HGR121, HGR123, HGR124 and HGR126, maintained their intracellular FD content after a shift to 27°C, suggesting that both the influx and efflux processes were affected to the same extent at this temperature giving rise to a "frozen" phenotype. Nevertheless, their plateau decreased when the cells were incubated at 29°C, showing that in these lines the endocytic process remains more sensitive to elevated temperature than the exocytic process.

**DISCUSSION**

We used random mutagenesis to generate Dictyostelium discoideum pinocytosis mutants which we enriched with multiple rounds of selection using our novel photodynamic killing method [10]. All our clones were tsg and had fluid-phase endocytosis activities affected at restrictive temperature (27°C). Some of them were completely blocked whereas others were still able to maintain a minimal activity. In all cases, pinocytosis was rapidly and reversibly impaired, while ATP levels were stable throughout. The decrease of endocytosis was likely to be the primary defect in these cells. Growth arrest could be considered as a secondary consequence of reduced nutrient uptake.

The initial loss of endocytic functions could be due either to the heat inactivation of a properly folded protein with a slow turnover or to the heat-induced misfolding of a rapidly turned-over protein. In both cases the time-course of pinocytosis inhibition is dependent on the amount of pre-existing active protein as well as the speed of heat inactivation of each individual protein. This issue cannot be addressed at this point but other experiments using the protein synthesis inhibitor cycloheximide showed that proteins with rapid turnover could be required for pinocytosis activity in Dictyostelium [7]. A similar screen for thermosensitive endocytosis mutants was performed with a fluorescent-activating cell sorting apparatus [1].

One strain (Indy1) has been studied in detail and its phenotype compared to that of HGR102, HGR105 and HGR110 (growth and pinocytosis rapidly but reversibly stopped at 27°C). In contrast, our other mutants showed a transient recovery of pinocytosis activity. This phenotype could be due to the induction of either heat-shock proteins or of an alternative fluid uptake mechanism. This last alternative was shown to occur in HeLa cells overexpressing mutant dynamin [5].

Dictyostelium discoideum amoebae provide an excellent model for pinocytosis studies. We provide a new set of mutants to genetically dissect the endocytosis pathway. We found that these mutants have interesting thermoconditional phenotypes in which entry and exit of fluids were uncoupled. The technique of choice to investigate the specific defects would be the transformation with a library of wild type DNA. However, this approach awaits improved transformation procedures which would allow the routine screening of whole genomic libraries in Dictyostelium. These mutants might also be useful for biochemical studies and for in vitro vesicular fusion assays [11].

Ultimately, we hope to discover new cytosolic or membrane bound proteins involved in vesicular traffic of cells.

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