A Chinese Hamster Ovary Cell Mutant with a Heat-sensitive, Conditional-lethal Defect in Vacuolar Function

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ABSTRACT   We describe a mutant derived from Chinese hamster ovary cells that is heat-sensitive for viability and for resistance to certain protein toxins. This mutant, termed G.7.1, grows normally at 34°C but does not grow in Dulbecco's modified Eagle's medium at 39.5°C. However, when this medium is supplemented with $FeSO_4$, the mutant cells will grow at the elevated temperature. At 39.5°C, G.7.1 cells acquire resistance to diphtheria toxin, modeccin, and Pseudomonas aeruginosa exotoxin A, all of which are protein toxins that require endocytosis and exposure to a low pH within vesicles before they can invade the cytosol and kill cells. The properties of mutant G.7.1 could result from a heat-sensitive lesion that impairs vacuolar acidification. We assayed the ATP-stimulated generation of pH gradients across the membrane of vesicles in cell-free preparations from mutant and parental cells by the partitioning of acridine orange into acidic compartments and found that the acidification response of the mutant cells was heat-labile. Altogether the evidence suggests that G.7.1 cells contain a heat-sensitive lesion that impairs vacuolar acidification and that they fail to grow in normal medium at 39.5°C because they cannot extract Fe^{+3} from transferrin, a process that normally requires exposing transferrin to a low pH within endosomal vesicles.

Endocytosis is the complex process by which most eucaryotic cells actively internalize portions of their plasma membrane in the form of vesicles (1). Extracellular ligands that bind to cell surface receptors are efficiently captured and sequestered within endocytic vesicles if the receptors are included in the internalized area of the plasma membrane. Potential subcellular destinations for endocytosed material vary and include delivery to the degradative lysosomal compartment, recycling back to the plasma membrane, and delivery to vacuoles in the Golgi region of the cell. An intermediate station in this traffic pattern is the endosome (2), also called the CURL (compartment of uncoupling of receptor and ligand) (3) and the receptosome (4), which receives material from the initial endocytic vesicles. It is now established that the pH within endosomes is acidic (5–7) and that the low pH is important in diverting some ligands to their appropriate destinations in the cell (2). The molecular mechanisms underlying the movement and sorting of endocytosed material are poorly understood.

Endocytosis normally functions to internalize extracellular fluid, plasma membrane, and physiologically important ligands; however, certain viruses (8) and protein toxins (9) take advantage of the endocytic pathway to invade the interior of eucaryotic cells. Many protein toxins, for example, are internalized by receptor-mediated endocytosis and then escape from vesicles to enter the cytosol where an enzymatic activity associated with a toxin inactivates an essential cell function, efficiently killing the cell. Because the effects of the toxins are so lethal, they offer the opportunity to search for toxin-resistant mutants that are defective in endocytic function. Several groups have reported isolating such mutants. Robbins et al. (10) isolated a mutant, selected for resistance to diphtheria toxin, that was pleiotropically defective in receptor-mediated endocytosis. Moehring and Moehring (11) have also described a class of mutants, called DPV^r mutants (resistant to diphtheria toxin, Pseudomonas aeruginosa exotoxin, and certain RNA viruses), that were shown by Merion et al. (7) to be defective in endosomal acidification. Ray and Wu (12) reported a mutant that was resistant to Pseudomonas aeruginosa exotoxin and the plant toxin ricin and that apparently is defective in endocytosing these two toxins. Many defects in endocytic function, however, are likely to be lethal and to isolate mutants carrying such deleterious lesions it will be necessary to devise selection procedures that can rescue con-

ditional-lethal mutants. We report here the use of protein toxins to isolate a mutant of Chinese hamster ovary (CHO)[1] cells that carries a conditional-lethal defect in endocytic function. The mutant was selected by exposing cells simultaneously to two toxins, diphtheria toxin (DT; M_r 58,342) and the plant toxin modeccin ($M_r \sim 66,000$).

There are four steps in the mechanism of DT action: (a) Binding of the toxin to cell surface receptors (13). The receptor is not well characterized but may be a cell surface glycoprotein (14, 15). (b) Internalization of the toxin via endocytosis into an acidic compartment, presumably endosomal vacuoles (16, 17). (c) Insertion of the toxin into the membrane in response to a conformational change caused by the low pH. As a result of insertion, part of the toxin (fragment A, $M_r = 21,167$) is apparently thrust through the membrane into the cytoplasm (18–22). (d) Fragment A is an enzyme that transfers the ADP-ribosyl moiety of nicotinamide adenine dinucleotide (NAD) to elongation factor 2 (EF-2) in the cytosol, inactivating EF-2 and thus blocking protein synthesis (13).

The mechanism of modeccin action is not as well characterized as that of DT but several of the steps are known: (a) Modeccin binds to cell surface receptors containing galactose residues and is internalized into vesicles (9, 16). (b) A low pH within the vesicles is apparently needed for part of modeccin, the A chain ($M_r \sim 30,000$), to reach the cytosol, but the mechanism of transport through a membrane is not yet known. The importance of a low pH is indicated by the fact that drugs that elevate the pH within normally acidic vesicles prevent the A chain from reaching the cytosol (16, 23). There is also evidence that modeccin, unlike DT, does not pass to the cytosol from prelysosomal vesicles but that modeccin might traverse a postendosomal compartment, perhaps lysosomes, en route to the cytosol (24, 25). (c) Once in the cytosol, the modeccin A chain arrests protein synthesis by enzymatically inactivating the 60S ribosomal subunit (9).

We selected mutants by exposing cells to both DT and modeccin, as opposed to using a single toxin, to avoid isolating mutants that were impaired in toxin binding or that contained a toxin-resistant cytosolic target. Since DT and modeccin initially interact with different cell surface receptors, it is improbable that a mutant resistant to both toxins would be defective at the level of toxin binding. Because DT and modeccin arrest protein synthesis by inactivating different gene products in the cytosol, it is also improbable that a mutant resistant to both toxins would express resistance at the level of either EF-2 or 60S ribosomal subunits. A mutant resistant to both toxins will most likely contain a lesion affecting a common event required either for the internalization of toxin-receptor complexes into vesicles or the passage of the enzymatically active chains of endocytosed toxins from inside vesicles out to the cytosol. The mutant described here appears to be in the latter category and the available evidence suggests that the mutant carries a heat-sensitive lesion that impairs the acidification of endocytosed material.

MATERIALS AND METHODS

Materials: DT was purchased from Connaught Laboratories (Willodale, Ontario, Canada) and purified by ion exchange chromatography (26). Modeccin

was obtained from Pierce Chemical Corp (Rockford, IL). *Pseudomonas aeruginosa* exotoxin A (PE) was the generous gift of Dr. S. Leppla (U. S. Army Medical Research Institute of Infectious Diseases, Frederick, MD). Abrin was purchased from Sigma Chemical Corp (St. Louis, MO). Rabbit antimodeccin and anti-DT were prepared as previously described (24, 26). Sources for other reagents and for cell culture supplies have been previously identified (17, 24, 27, 28).

Cell Culture Conditons: The CHO cell line, CHO-K1, was obtained from the American Type Culture Collection (Rockville, MD). The G.7.1 cell strain was derived from this line as described in Fig. 1. The cells were maintained in Dulbecco's modified Eagle's medium (DME) supplemented with 5% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.02 M HEPES, 4.5 g/L glucose, and 4 g/L proline. The cells were grown in a humidified 10% CO_2 and 90% air atmosphere at 34°C unless otherwise indicated. For protein synthesis assays, cells were plated in 24-well Falcon plates (Falcon Labware, Oxnard, PA) at an approximate density of 1×10^5 cells/well in 1 ml of growth medium and grown overnight at 34°C. The cultures were then either maintained at 34°C or elevated to 39.5°C for 48 h prior to initiating an experiment. Protein synthesis was assayed by the incorporation of L-[4,5-³H]leucine into trichloroacetic acid-precipitable material as previously described (17, 24, 27). The assay medium used in leucine incorporation experiments was growth medium that contained 1/100th the normal amount of leucine.

Growth Curves and Plating Efficiency Experiments: To initiate a growth curve experiment, $\sim 5 \times 10^3$ cells suspended in 1 ml of growth medium or growth medium supplemented with 3.0 μM $FeSO_4$ were inoculated in each well of a 24-well Falcon plate and grown overnight at 34°C. The following day the plates were either maintained at 34°C or shifted to 39.5°C. The cells were harvested by trypsin treatment at the times indicated and counted with a Coulter Counter (Coulter Electronics, Hialeah, FL). Plating efficiencies were determined by plating cells overnight at 34°C in 25-cm² tissue culture flasks in 5 ml of growth medium. The flasks were either maintained at 34°C or shifted to 39.5°C as described in the figure legends. 2 wk later the cultures were stained with 0.5% crystal violet and the colonies were counted.

Preparation and Use of Radiolabeled Modeccin and DT: ¹²⁵I-modeccin was prepared as described by Draper et al. (24). The specific activity of recovered modeccin ranged between 3 and 23×10^6 cpm/μg for different preparations. Cells were incubated with ¹²⁵I-modeccin as described in figure legends. Acid-soluble radioactivity in the medium was measured by mixing 0.5 ml of medium with an equal volume of cold 10% (wt/vol in water) trichloroacetic acid in a microfuge tube. After 20 min, the mixture was centrifuged for 5 min and the radioactivity in the supernatant was assayed with a Beckman model 5500 gamma counter (Beckman Instruments, Palo Alto, CA). Galactose, which inhibits the binding of modeccin to cells (9), was included in the medium of controls to assess the extent of nonspecific binding. Cell-associated radioactivity was assayed by gamma counting after cells were dissolved in 1.0 N NaOH.

DT was radiolabeled by the chloramine T method as described by Marnell et al. (17). The specific activity of the toxin was 2×10^7 cpm/μg. Cells were exposed to ¹²⁵I-DT as described in the legend to Fig. 7. Cell-associated radio-
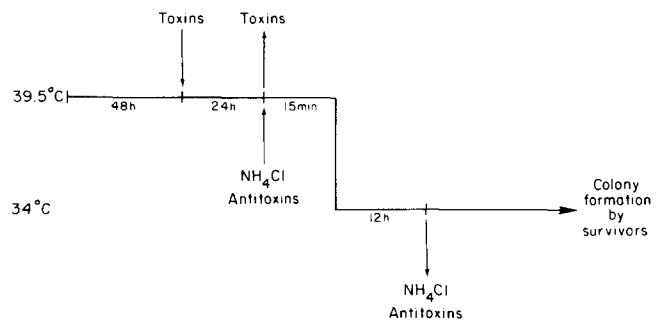
FIGURE 1 Protocol for the selection of toxin resistant, heat-sensitive mutants. CHO cells were mutagenized at 34°C by exposure to ethylmethane sulfonate (250 μg/ml) for 24 h and then grown for 10 d at 34°C. The cells were placed at 39.5°C for 48 h prior to the addition of modeccin (10^{-10} M) and DT (10^{-9} M). After a 24-h incubation at 39.5°C, the toxin-containing medium was removed and replaced with medium containing 20 mM NH_4Cl, antimodeccin serum (8% vol/vol), and antidiphtheria toxin serum (2% vol/vol). After 15 min, the cells were shifted to 34°C. 12 h later the NH_4Cl and antitoxins were removed. The cultures were then maintained at 34°C to allow the growth of surviving cells.

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activity was assayed by gamma counting after cells were dissolved in 1.0 N NaOH. Nonspecific accumulation was determined in parallel controls that had received radiolabeled DT in the presence of a 100-fold excess of unlabeled toxin. Data in experiments with radiolabeled toxins are the average of two samples.

Acridine Orange Assays for Vesicle Acidification: The rate of acridine orange accumulation within acidic vesicles was measured as the difference between the absorbance at 492 and 540 nm with an Aminco-Chance dual-wavelength spectrophotometer as described by Stone et al. (28). The acidification buffer, adjusted to pH 7.0, contained 30 mM histidine, 130 mM NaCl, 20 mM KCl, 2 mM $MgCl_2$, and 2.5 μM acridine orange. Additions to this buffer were as described in figure legends. Cells were harvested by trypsinization and homogenized at 4°C with a Dounce homogenizer in a buffer containing 10 mM HEPES, 0.25 M sucrose, 2 mM ethylene diamine tetracetic acid, pH 7.3. Postnuclear supernatants were prepared by centrifuging the homogenate at 800 g for 10 min.

RESULTS

The Selection Procedure

The protocol used in selecting the mutants is outlined in Fig. 1. Cells were mutagenized, grown at 34°C for 10 d and then placed at 39.5°C for 48 h to allow expression of heat-sensitive lesions. Both DT (10^{-9} M) and modeccin (10^{-10} M) were added for 24 h to kill sensitive cells. The cells were then washed and incubated in medium containing ammonium chloride (20 mM), anti-DT, and antimodeccin at 34°C to inactivate cell-associated toxin molecules. After 24 h the inactivating medium was replaced with fresh growth medium and the cells were incubated at 34°C for 5 wk to allow surviving cells to form colonies. The treatment to inactivate the toxins is essential to prevent any cell-associated toxin molecules that are arrested *en route* to the cytosol by a lesion at the nonpermissive temperature from killing a mutant upon recovery at the permissive temperature. None of our previous attempts to isolate heat-sensitive mutants without the inactivation step were successful. The antitoxins neutralize any toxin molecules on the cell surface. The ammonium chloride protects cells from both DT and modeccin by elevating the pH within acidic vesicles. Moreover, previous work with DT and modeccin had suggested that exposing cells to antitoxins in the presence of ammonium chloride also resulted in the inactivation of toxin molecules already endocytosed as long as the internalized toxins had not yet been exposed to a low pH (16, 26, 27).

From the selection experiment outlined in Fig. 1 we isolated two mutants that are heat-sensitive for viability from an initial population of 2 × 10⁸ mutagenized cells. One of the mutants, G.7.1, is further characterized in this report.

Growth Properties of G.7.1

The growth rate of G.7.1 cells at 34 and 39.5°C is compared with parental cells in Fig. 2. At 34°C, the mutant cells grew at the same rate as the parental cells. At 39.5°C, the mutant cells ceased to grow after ~25 h but the cells did not begin to die and detach from the bottom of the culture plates until ~125 h after elevating the temperature. Not shown in the time frame of Fig. 2 is that the cells did appear to die *en masse* and detach from the dishes after more than a week at 39.5°C. Although the mutant cells eventually died at 39.5°C, they nevertheless remained metabolically active for at least 72 h after being placed at 39.5°C. This was indicated by the fact that they synthesized protein at a rate comparable with that of normal cells during this period (data not shown).

The plating efficiencies of mutant and parental cells at 34°C and 39.5°C are shown in Table I. At 34°C, the mutant cells
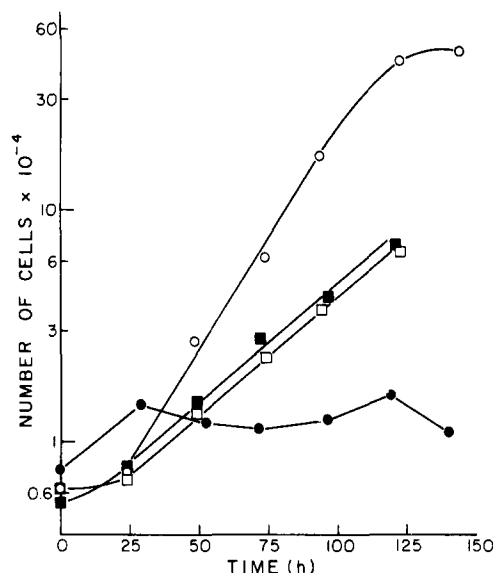
FIGURE 2   Growth of parental and G.7.1 cells at 34°C and 39.5°C. Parental cells (O, □) and G.7.1 cells (●, ■) were inoculated in 24-well culture plates at a density 5 × 10³ cells per well and incubated overnight at 34°C. The following day, one set of parental cells (O) and one set of G.7.1 cells (●) were placed at 39.5°C. At the indicated times, cells were harvested from triplicate wells and the number of cells determined with a Coulter counter.

TABLE I

Plating Efficiencies of Parental and G.7.1 Cells at 34 and 39.5°C

Cell type	No. of cells plated	Plating efficiency (%)	
		34°C	39.5°C
Parental	300	69	37
G.7.1	300	18	0
G.7.1	5,000	—	0

Parental or mutant cells were inoculated at the indicated numbers in 25-cm² culture flasks containing growth medium and placed at 34 or 39.5°C for 2 wk. Colonies were then stained with 0.5% crystal violet and counted. Plating efficiencies are the average of three samples and are expressed as the number of colonies that grew with respect to the number of cells that were inoculated.

formed colonies, although at reduced efficiency, compared with parental cells. At 39.5°C, the plating efficiency of the parental cells was somewhat reduced while the mutant cells did not form colonies, even when as many as 5,000 cells were plated. To see how long an incubation at 39.5°C was necessary to kill the mutant cells, the cells were plated and allowed to attach to culture dishes overnight at 34°C. The cultures were then placed at 39.5°C and samples were removed at different times to measure the number of colonies formed at 34°C. As seen in Fig. 3, the survival of G.7.1 cells declined rapidly after 24 h at 39.5°C.

Response of G.7.1 to Various Protein Toxins

The concentrations of modeccin and DT required to inhibit protein synthesis by 50% (IC50) in parental and mutant cells exposed to these toxins for 3 h at either 34 or 39.5°C are shown in Table II. For assays at 39.5°C, the cells were incubated at the elevated temperature for 48 h prior to toxin addition because control experiments revealed that at least a 24-h incubation at 39.5°C was required to fully express the toxin-resistant phenotype. As seen in Table II, the mutant cells were slightly more resistant to both toxins at 34°C than the parental cells. At 39.5°C, the mutant cells acquired great
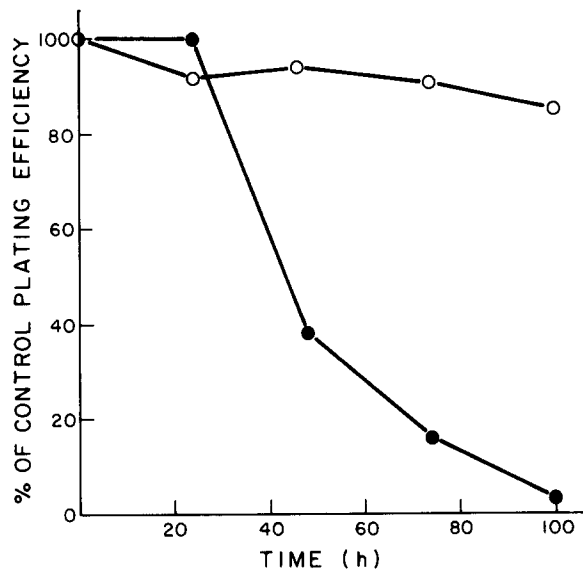
FIGURE 3 Survival of parental and G.7.1 cells in plating efficiency tests as a function of time at 39.5°C. 300 cells were inoculated in 25-cm² cell culture flasks containing growth medium and grown overnight at 34°C. The following day, experimental cultures were shifted to 39.5°C and at the indicated times flasks of each cell type were returned to 34°C. 2 wk later the cultures were stained with 0.5% crystal violet and the number of colonies were counted. Each point is the average of colonies from two flasks and the data is expressed as the percentage of colonies on control plates that were maintained at 34°C for each cell type. O, parental cells; ●, G.7.1 cells.

TABLE II

Sensitivity of Parental and G.7.1 Cells to Protein Toxins at 34 and 39.5°C

| | IC50 (nM) at 34°C | | | IC50 (nM) at 39.5°C | | |
Toxin	Paren-tal	G.7.1	IC50 G.7.1/ IC50 parental	Paren-tal	G.7.1	IC50 G.7.1/ IC50 pa-rental
Modeccin	0.05	0.2	4	0.02	>100	>5,000
DT	3	9	3	0.3	4	13
PE	2	2	1	0.05	0.5	10
Abrin	0.3	0.3	1	0.1	0.03	0.3

Parental and G.7.1 cells were inoculated in 24-well culture plates and incubated overnight at 34°C. The following day, half of the cultures were placed at 39.5°C for 48 h. Cells were then exposed at both temperatures to different concentrations of modeccin or diphtheria toxin (DT) for 3 h and Pseudomonas aeruginosa exotoxin A (PE) or abrin for 6 h. During the last hour of each incubation, L-[4,5-³H]leucine was added and the amount of radioactivity incorporated into acid-insoluble material was measured. The IC50 is the concentration of a toxin in nanoMolars required to inhibit protein synthesis by 50% as compared with controls that received no toxin.

resistance to modeccin and, to a lesser extent, resistance to DT.

Also shown in Table II are IC50 values after a 6-h exposure to two other toxins, Pseudomonas aeruginosa exotoxin A (PE) and the plant toxin abrin. PE is similar to DT in that a catalytic activity associated with the toxin inactivates EF-2 in the cytosol by a mechanism that appears identical to that of DT (29). Moreover, PE requires endocytosis (30) and acidification within vesicles (11) to reach the cytosol. The cell surface receptors for DT and PE are, however, different (31). Abrin is similar to modeccin in that it inactivates 60S ribosomal

subunits and requires endocytosis for the enzymatic subunit of the toxin to reach the cytosol (9, 16). Unlike the other three toxins, however, abrin does not require a low pH within the vacuolar compartment to invade the cytosol; in fact, abrin is more cytotoxic to cells in the presence of drugs that elevate vacuolar pH (9). As seen in Table II, the mutant cells acquired a tenfold resistance to PE at 39.5°C, but they became hypersensitive to abrin at the elevated temperature.

Interaction of Radiolabeled Modeccin with Cells

One explanation for the resistance of the mutant cells to several toxins at 39.5°C is that the cells no longer bind the toxins at the nonpermissive temperature. Therefore, we compared the binding of radiolabeled modeccin to mutant and parental cells. To prevent the endocytosis of modeccin during the experiment, the cells were chilled to 4°C during the binding assay. Because binding was measured at 4°C, it was necessary to check whether the mutant cells returned to a modeccin-sensitive state as a result of chilling. This was assessed in controls by measuring the response of G.7.1 cells to modeccin after the cells were incubated at 39.5°C, chilled for 1 h to 4°C, and then immediately treated with modeccin at 39.5°C for 3 h before measuring the effect of the toxin on protein synthesis. The mutant cells were still ~5,000-fold more resistant to modeccin than parental cells after this manipulation. This suggested that chilling the cells did not induce modeccin sensitivity because at least 24 h were usually required for cells to go from a modeccin-sensitive phenotype at 34°C to full modeccin resistance upon elevating the temperature. To measure modeccin binding, cells were incubated for 48 h at 39.5°C, chilled to 4°C, and exposed for 1 h to different concentrations of ¹²⁵I-modeccin. Unbound modeccin was washed away and cell-associated radioactivity was measured. As seen in Fig. 4, there was no significant difference in the amount of modeccin bound to mutant and parental cells. We did not use concentrations of modeccin in this experiment sufficient to reach saturation because only limited supplies of the toxin were available.

A second explanation for the resistance of the mutant cells to toxins is that the cells do not endocytose bound toxin at 39.5°C. To check this, we compared the internalization of modeccin by parental and mutant cells at elevated temperature. Cultures were placed at 39.5°C for 48 h to induce the lesion in G.7.1 cells, chilled to 4°C, incubated with ¹²⁵I-modeccin, washed, and returned to 39.5°C. At different times, the cells were again chilled, washed with medium containing 0.1 M galactose to remove modeccin remaining on the cell surface, and the internalized modeccin was measured. As seen in the inset of Fig. 4, there was no difference between parental and G.7.1 cells in the amount of modeccin internalized up to 1 h after cells containing modeccin on their surface were placed at 39.5°C. Therefore, the lesion in G.7.1 cells appears not to affect the initial internalization of receptor-bound modeccin into endocytic vesicles.

Also apparent from the inset of Fig. 4 is that the amount of modeccin associated with the parental cells began to decline after 1 h, indicating that some of the internalized modeccin was excreted back into the medium; however, the amount of modeccin associated with G.7.1 cells did not decline. This suggested that the excretion of degraded modeccin was impaired in the mutant cells. To test this, parental and G.7.1 cells were incubated at the nonpermissive temperature with ¹²⁵I-modeccin to allow internalization, washed to remove
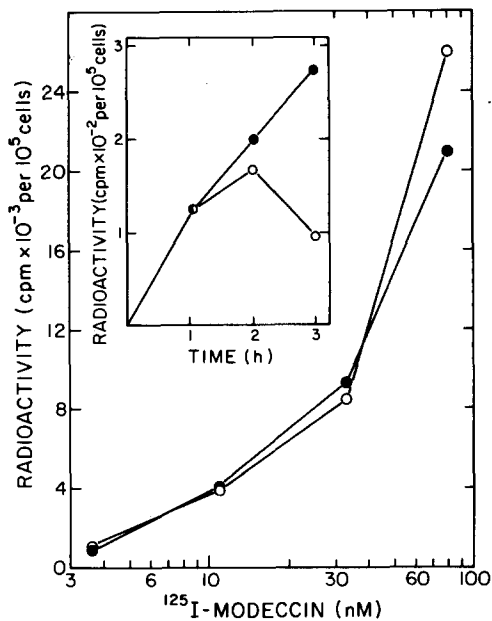
FIGURE 4 Binding and internalization of ^{125}I-modeccin by parental and G.7.1 cells at 39.5°C. To measure modeccin binding, parental (O) and G.7.1 (●) cells were inoculated in 24-well culture dishes, grown overnight at 34°C, and placed at 39.5°C for 48 h. The cells were then placed at 4°C in DME without serum, but containing 1 mg/ml BSA, and exposed for 1 h to the indicated concentrations of ^{125}I-modeccin. Parallel controls received ^{125}I-modeccin in the presence of 0.1 M galactose to assess nonspecific binding. The cells were then washed free of unbound material and assayed for radioactivity. Specific binding is plotted on the ordinate. Nonspecific binding was, at most, 60% of the total bound material (this was at the lowest modeccin concentration). The specific activity of the ^{125}I-modeccin was 2.3 × 10^7 cpm/μg. (Inset) To measure internalization of modeccin, parental (O) and G.7.1 (●) cells were grown as above and incubated at 4°C with 10 nM ^{125}I-modeccin in the presence or absence of 0.1 M galactose. The cells were then washed to remove unbound material and placed in medium at 39.5°C. At the indicated times, the cells were chilled to 4°C and incubated for 1 h in medium containing 0.1 M galactose to remove ^{125}I-modeccin from the cell surface. The cells were then washed and assayed for radioactivity. Internalized radioactivity deriving from specifically bound modeccin is plotted on the ordinate. Radioactivity deriving from nonspecifically bound modeccin was, at most, 59% of the total internalized counts. The specific activity of the ^{125}I-modeccin was 4.7 × 10^6 cpm/μg.

modeccin in the medium, and the acid-soluble radioactivity appearing in the medium upon further incubation at 39.5°C was determined. As shown in Fig. 5, the mutant cells excreted less digested toxin than parental cells.

Effect of Acidic Medium on the Cytotoxic Activity of DT with Mutant G.7.1

When cells bearing DT on their exterior surface are exposed to culture medium at pH 4.8, the toxin penetrates directly through the plasma membrane into the cytosol (18, 26, 27, 32). This provides a functional test for the presence of DT receptors on the cell surface. To assay for toxin binding, mutant and parental cells at 34 or 39.5°C were exposed to different concentrations of DT for 2 h to allow the toxin to associate with cells and then the cells were washed to remove unbound toxin. Medium at pH 4.8 was added for 10 min and then replaced with medium at neutral pH. Protein synthesis

was assayed 3 h later. Ammonium chloride was present in the medium during this experiment to block the passage of any endocytosed toxin molecules from inside vesicles out to the cytosol. As seen in Fig. 6, both mutant and parental cells were equally sensitive to the toxin under these conditions. This is evidence that G.7.1 still retains DT receptors at the nonpermissive temperature. Moreover, there is apparently nothing wrong with the plasma membrane of the mutant cells that blocks the penetration of fragment A through the membrane into the cytosol as long as a low pH is provided.

Accumulation of Radiolabeled DT by Mutant and Parental Cells

CHO cells appear to have few DT receptors and we were unable to satisfactorily extract data on the specific binding of radiolabeled DT to surface receptors at 4°C from the high background of nonspecifically bound material (although see reference 33). We were able to obtain good data on the specific accumulation of radiolabeled toxin by cells over longer incubations at physiological temperatures. Mutant and wild-type cells were incubated at either 34 or 39.5°C for 48 h and exposed to ^{125}I-DT with or without an excess of unlabeled toxin. The specific accumulation of DT by the cells over a 6 h period is seen in Fig. 7. At 34°C the mutant and wild-type cells accumulated similar amounts of toxin. At 39.5°C, the initial rate of toxin accumulation in the mutant was about the same as in the parental cells but by 6 h significantly more toxin was accumulated by the mutant than by either the parental cells or by the mutant at 34°C.

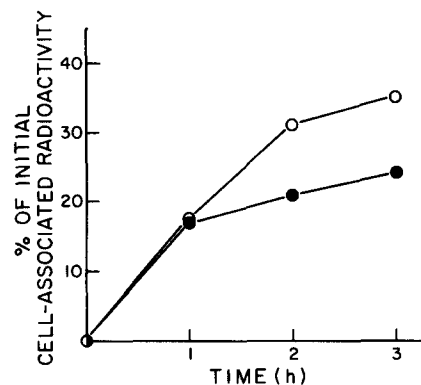The accumulation of DT by cells represents the difference between the rate of receptor-mediated internalization of the

FIGURE 5 Excretion of degraded ^{125}I-modeccin by parental and G.7.1 cells at 39.5°C. Parental (O) and G.7.1 (●) cells were grown overnight in 24-well culture dishes and placed at 39.5°C for 48 h. The cells were then incubated at this temperature with medium containing 10 nM ^{125}I-modeccin for 1.5 h to allow binding and internalization of modeccin. The cells were washed to remove modeccin in the medium and fresh medium at 39.5°C was added. At this time there were an average of 6,200 specific cpm associated with 10^5 parental cells and 6,000 specific cpm associated with 10^5 G.7.1 cells. At the times indicated, the acid-soluble radioactivity appearing in the medium was determined. Parallel controls received ^{125}I-modeccin in the presence of 0.1 M galactose to assess acid-soluble radioactivity deriving from nonspecifically internalized modeccin. This was, at most, 41% of the total at any point. Acid-soluble radioactivity in the medium deriving from specifically internalized modeccin is plotted on the ordinate as the percent of initial cell-associated modeccin. The specific activity of the ^{125}I-modeccin was 7.6 × 10^6 cpm/μg.
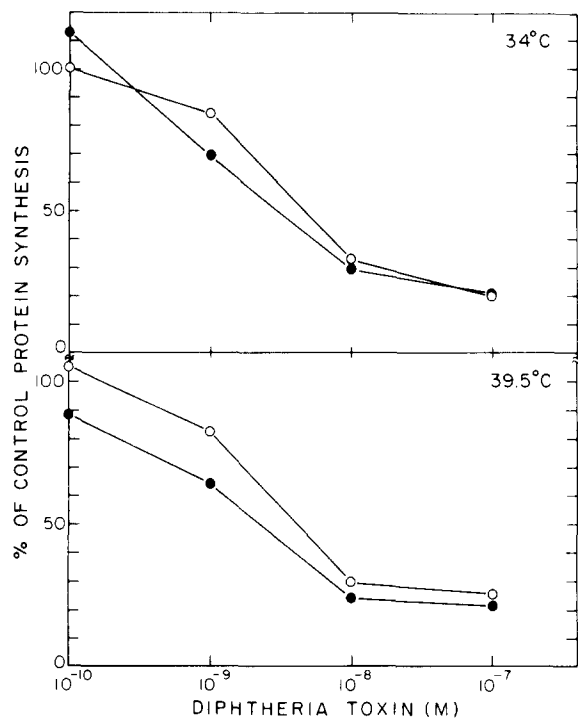
FIGURE 6 The response of parental and G.7.1 cells to diphtheria toxin after toxin-treated cells were exposed to acidic culture medium. Parental (O) and G.7.1 (●) cells were grown overnight in 24-well culture dishes at 34°C and then samples of each cell type were placed at 39.5°C for 48 h. Subsequent manipulations were at either 34°C (top) or 39.5°C (bottom). Fresh medium containing 20 mM NH$_4$Cl was added and 15 min later the indicated concentrations of toxin were added for 2 h. The medium was replaced with medium at pH 4.8 containing 20 mM NH$_4$Cl and 10 min later this was replaced with assay medium at norml pH containing 20 mM NH$_4$Cl. After 2 h, L-[4,5-^{3}H]leucine (2 μCi/ml) was added for 1 h and the amount of acid-insoluble radioactivity was determined. The data is expressed as the percentage of radioactivity in parallel controls that received no toxin.

toxin and the rate at which toxin degradation products are excreted (34). That the mutant cells accumulated even more toxin at the nonpermissive temperature than the wild-type cells suggests that the resistance of G.7.1 cells to DT cannot be explained by a decrease in the rate of receptor-mediated internalization of the toxin. This is consistent with the results of Fig. 6 that the mutant cells express functional DT receptors at 39.5°C. The over accumulation of ^{125}I-DT by G.7.1 cells suggests that the degradation and excretion of DT is impaired, as was also observed with modeccin.

G.7.1 Cells Grow in the Presence of Added Iron at 39.5°C

The properties of the mutant cells suggest that they are not defective in toxin binding or internalization at 39.5°C and it is likely, therefore, that the escape of the catalytic subunits of the toxins from vesicles to the cytosol is impaired. Since G.7.1 cells were only resistant to toxins that required a low pH for activity, the mutant cells might express a lesion at 39.5°C that impairs vacuolar acidification. One anticipated consequence of a defect in the acidification of endocytosed material would be a disruption in means by which cells acquire iron via the transferrin cycle: Diferric transferrin binds to cell surface

receptors and is endocytosed into an acidic prelysosomal compartment, presumably the endosomes, where the low pH causes the bound ferric ions to dissociate. Apotransferrin is then recycled back to the cell surface where it dissociates to bind more iron and reinitiate the cycle (35–37). Unless the transferrin encounters a low pH, the iron cannot be extracted from the transferrin. If the mutant cells are acidification-defective at 39.5°C, they may die from iron starvation; therefore, we measured the growth of the cells in the presence of increased exogenous FeSO$_4$ (DME normally used in our studies contains only 0.2 μM Fe^{+3}). As shown in Fig. 8, the mutant cells grew, although slowly, when 3.0 μM FeSO$_4$ was present in the medium. The supplemented medium did not support colony formation by the mutants at high temperature but the cells did plate, although at low efficiency (~3%), in Coons modification of Ham's F-12 medium, a rich medium that contains 3 μM FeSO$_4$. These data suggest that the mutant cells become auxotrophic for iron at 39.5°C. In medium with supplemental iron the mutant cells were still resistant to modeccin at 39.5°C (data not shown), indicating that the presence of iron did not reverse the toxin-resistant phenotype.

Acidification of Subcellular Vesicles from G.7.1 Cells is Heat-labile

To assay the generation of a proton gradient across the membrane of subcellular particles, we measured the ATP-stimulated partitioning of acridine orange into vesicles with cell-free preparations from mutant and parental cells. Acridine orange is a weak base that accumulates inside acidic vesicles. The accumulation quenches the absorbance of the dye at 492 nm, which is readily detected with a dual-wavelength spectrophotometer as a decrease in the difference between the absorbance at 492 and 540 nm. The initial rate of
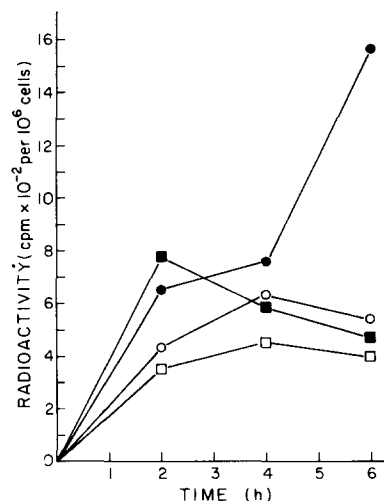

FIGURE 7 The accumulation at 34°C and 39.5°C of ^{125}I-diphtheria toxin by parental and mutant cells. Parental (□, ■) and G.7.1 (O, ●) cells were inoculated into 100-mm culture dishes and grown overnight at 34°C. The next day, half the cultures were elevated to 39.5°C for 48 h. ^{125}I-diphtheria toxin (100 ng/ml) was added and at the indicated times the cells were washed and assayed for ^{125}I by gamma counting. Parallel controls received ^{125}I-diphtheria toxin in the presence of a 100-fold excess of unlabeled toxin to determine the extent of nonspecific accumulation. Specific accumulation is plotted on the ordinate. Open symbols, 34°C. Closed symbols, 39.5°C.
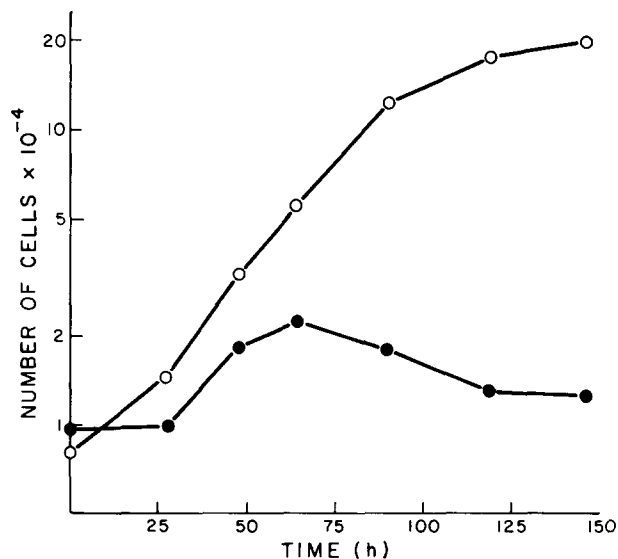
FIGURE 8 Growth of G.7.1 cells in medium supplemented with FeSO₄ at 39.5°C. G.7.1 cells were inoculated at a density of 5 × 10³ cells per well in 24-well culture dishes in growth medium (●) or growth medium supplemented with 3.0 μM FeSO₄ (○) and grown overnight at 34°C. The following day, cultures were moved to 39.5°C and at the indicated times the cells were harvested from three wells and the number of cells were determined with a Coulter counter. The addition of 3.0 μM FeSO₄ to DME did not affect the growth rate of parental cells (data not shown).

acridine orange accumulation has been recently used as a semiquantitative assay for the rate of ATP-dependent proton gradient formation in vesicles from turtle urinary bladder (38), Golgi-derived vesicles (39), and clathrin-coated vesicles (28). Shown in Fig. 9 is the rate at which the 492-nm signal of acridine orange was quenched upon addition of ATP to a postnuclear supernatant from parental cells. Monensin, a carboxylic ionophore that exchanges protons for Na^+ across membranes, released the acridine orange from vesicles, indicating dissipation of the pH gradient. The protonophore carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone (FCCP) had an effect similar to that of monensin (data not shown). The initial rate in the decline of the 492-540-nm signal in several postnuclear supernatant preparations from both parental and G.7.1 cells grown at 34°C was in the range of .001 OD units per minute per mg protein.

Also shown in Fig. 9 is the effect of N-ethyl maleimide (NEM), oligomycin, and sodium vanadate on the accumulation of acridine orange. Formation of a pH gradient was sensitive to NEM and resistant to oligomycin. This is good evidence that vesicles formed from inverted inner mitochondrial membranes are not responsible for the acidification activity observed in Fig. 9 because the proton translocating F_1-F_0 ATPase of the inner mitochondrial membrane is sensitive to oligomycin and resistant to NEM (28, 39). That vanadate did not inhibit acidification suggests that a phosphorylated protein intermediate is not involved in proton translocation. Sensitivity to NEM and resistance to oligomycin and vanadate are properties of proton translocating ATPases found in lysosomes (6, 40), endosomes (6), Golgi-derived vesicles (39), vesicles from turtle urinary bladder (38), and clathrin-coated vesicles (28).

We measured the initial rate of acridine orange quenching in extracts prepared from parental and G.7.1 cells that had

been shifted from 34 to 39.5°C for different times before the cells were harvested. As seen in Table III, the specific acridine orange quenching activity in extracts from G.7.1 cells had declined within 24 h to a plateau value while the activity in parental extracts was not significantly affected by growing the cells at 39.5°C. In this experiment, the G.7.1 cells (and parental controls) had been grown at 39.5°C in medium containing 3 μM FeSO₄ to minimize the effect on the results from any G.7.1 cells that would have died at 39.5°C if extra iron was not present in the medium. The data in Table III directly suggest that G.7.1 cells grown at 39.5°C become impaired in the acidification of intracellular vesicles, although not all acidification activity is lost. Also, the specific acidification response in extracts from G.7.1 cells harvested at 34°C was about one-half that in extracts from parental cells at 34°C. This is consistent with the finding that G.7.1 cells do express a slight resistance to DT and modeccin at 34°C (Table II).

As an additional test for the presence of a heat-sensitive lesion affecting the generation of proton gradients in extracts from G.7.1 cells, we measured the heat stability of the acidification activity upon incubating extracts at elevated temperature. Shown in Fig. 10 are heat-inactivation curves when postnuclear supernatants from parental and mutant cells
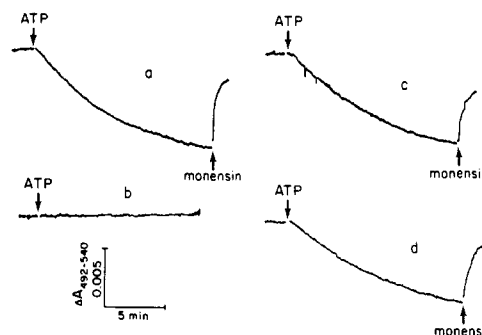

FIGURE 9 ATP-induced partitioning of acridine orange into acidic vesicles of postnuclear supernatants from parental cells. 50 μl of extract containing 800 μg of protein were added to 1.95 ml of acidification buffer at room temperature. The reactions were initiated by addition of ATP (1 mM). Proton gradients were collapsed by the addition of monensin (2.5 μM). In a, the acidification buffer contained no other additions. In b, N-ethylmaleimide (1 mM) was present; in c, oligomycin (1 μg/ml) was present and in d, Na₃VO₄ (100 μM) was present.

TABLE III

Initial Rates of Acridine Orange Accumulation in Extracts of Parental and G.7.1 Cells Grown at 39.5°C for Different Times

Time at 39.5°C	Initial rates of acridine orange accumulation (slope/mg protein)	
h	Parental	G.7.1
0	22.8 ± 1.1	11.5 ± 0.3
24	24.8 ± 1.9	3.3 ± 1.3
48	16.5 ± 4.6	2.3 ± 0.3
72	18.3 ± 8.8	3.8 ± 0.3

Parental or G.7.1 cells were grown on 150-mm dishes in DME supplemented with 3.0 μM FeSO₄ at 34°C and then shifted to 39.5°C for the indicated times. The number of cells plated was adjusted so that the final number of cells was ~2 × 10⁶ per dish at the time of harvesting and all plates were harvested and assayed the same day. Acidification assays were done with postnuclear supernatants as described in Materials and Methods. Each assay contained 200 μg of protein. Data is presented as the initial slope of acridine orange accumulation in arbitrary units per milligram protein. Assays were done in triplicate on each sample and the numbers represent the mean ± the standard deviation.
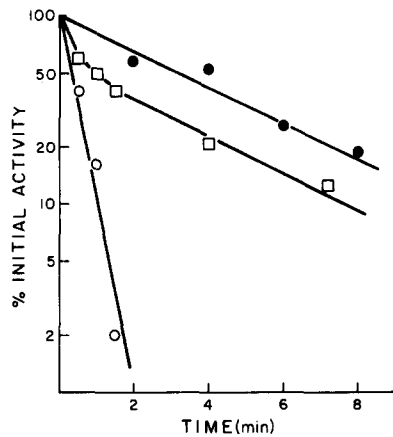
FIGURE 10 Heat-inactivation profiles of acidification activity in extracts of parental and mutant cells. Samples of postnuclear supernatants containing 800 μg protein from parental cells (●) or mutant cells (○) or a mixture containing 400 μg protein from both parental and mutant extracts (□) were heated to 56°C for the times indicated. Acidification activity was then measured at room temperature. Acidification activity was measured at room temperature as described in the legend of Fig. 8 except that the sensitivity of the spectrophotometer was increased fivefold. On the y-axis is plotted the percent of the initial activity before heating in terms of the initial rate of acridine orange quenching.

(grown at 34°C) were heated to 56°C for different times before measuring the initial rate of ATP-induced acridine orange uptake at room temperature. Activity in the extract from the mutant cells was reduced by 90% within 60 s at 56°C while more than 8 min at this temperature were required before a 90% loss in activity of the extract from parental cells. When extracts from mutant and parental cells were mixed and heated, the heat-inactivation curve indicated that mutant and parental components of the heterologous mixture were inactivated independently (Fig. 10). Thus, there is not some diffusible factor present in either extract that affects the heat inactivation of material in the other. These results demonstrate the presence of a highly heat-sensitive activity that affects either the formation or maintenance of a pH gradient in subcellular vesicles from mutant cells grown at 34°C.

DISCUSSION

A mutation might cause cross-resistance to several protein toxins by altering their cytoplasmic targets, EF-2 for DT and PE and 60S ribosomal subunits for modeccin. It is unlikely, however, that alterations in cytoplasmic targets could account for the toxin resistance of mutant G.7.1. First, it is highly improbable that two mutational events would have simultaneously occurred within the same cell to give heat-sensitive alterations in both EF-2 and the 60S ribosomal subunit. Second, the presence within the mutant cells of EF-2 that was sensitive to enzymatic inactivation by DT at the nonpermissive temperature was indicated by the observation that protein synthesis was equally reduced in parental and mutant cells at 39.5°C when fragment A of DT was artificially introduced into the cytosol through the plasma membrane of the cells in resonse to acidic culture medium. Thus, the defect in G.7.1 cells appeared to affect the delivery of the enzymatic subunits of the toxins to the cytosol.

A lesion might affect the delivery of the toxins to the cytosol at three levels of endocytic function: the initial toxin-receptor interaction, the internalization of toxin-receptor complexes

into vesicles, or the level at which the toxins pass out of the vacuolar compartment to enter the cytosol. Several lines of evidence suggest that G.7.1 is not defective in toxin binding or internalization into vesicles. At 39.5°C, the mutant and parental cells bound equivalent amounts of radiolabeled modeccin and the bound toxin was internalized at a similar rate by both cell types. The mutant and parental cells also accumulated radiolabeled DT at a similar rate up to 4 h after exposure to the toxin at the high temperature, suggesting that the receptor-mediated uptake of DT by the mutant cells was normal. The presence of DT receptors on the surface of the mutant cells at 39.5°C was further indicated by the observation that the cells were intoxicated when they were incubated with DT, washed, and exposed to acidic medium; if the cells had not bound DT, they should have been resistant to the toxin. Thus, there appears to be a lesion expressed at 39.5°C in G.7.1 cells that inhibits the passage of the enzymatic subunits of DT and modeccin (and presumably PE also) from vesicles to the cytosol after toxins have been endocytosed.

Several physiological properties of G.7.1 cells suggested that they carry a heat-sensitive lesion that impairs vacuolar acidification: (a) The mutant cells were only resistant to toxins that need a low vacuolar pH to invade the cytosol. (b) The cells were more sensitive to abrin (which does not require a low pH for cytotoxic activity) at the nonpermissive temperature than at 34°C. Hypersensitivity to abrin has been previously correlated with a drug-induced elevation in vacuolar pH (9). Moreover, the acidification-defective mutants isolated by Moehring and Moehring (11) were hypersensitive to ricin, a plant toxin with properties very similar to those of abrin (9). (c) Drugs such as ammonium chloride and monensin that elevate the pH within intracellular vesicles often impair lysosomal digestive capacity (1). A lesion that affected acidification of the toxins might also be expected to have an affect on the lysosomal digestion of the toxins and we found that the excretion of digested modeccin by G.7.1 cells was reduced at 39.5°C. The mutant cells also accumulated more ^{125}I-DT at 39.5°C then the parental cells, an observation consistent with the possibility that lysosomal digestion of DT was impaired in G.7.1 cells at elevated temperature. (d) Growth of G.7.1 at elevated temperature was restored by the presence of exogenous $FeSO_4$ in the culture medium. This suggests that the acquisition of iron by the mutant cells via transferrin was defective, which would be expected if transferrin were not exposed to a low pH within endosomes of the mutant cells at 39.5°C. Supporting this suggestion, we have recently found that the transferrin-mediated delivery of $^{59}Fe^{+3}$ to G.7.1 cells is impaired at 39.5°C even though the binding and endocytosis of transferrin by the cells is normal (manuscript in preparation). In the presence of extra iron, the mutant cells apparently acquire by nonspecific means sufficient iron to support some growth at 39.5°C.

Two lines of evidence directly suggested that ATP-stimulated pH gradient formation in subcellular vesicles from G.7.1 cells was heat-sensitive. First, the acidification activity in extracts from G.7.1 cells grown at 39.5°C was reduced compared with G.7.1 cells grown at 34°C or to parental cells grown at 39.5°C. Assuming that the in vitro acidification assay reflects the ability of G.7.1 cells to generate proton gradients in vivo at 39.5°C, then the heat-sensitive physiological properties of the mutant cells can be directly correlated with impaired acidification of vesicles in vivo. Second, the acidification activity in extracts from G.7.1 cells grown at 34°C was

highly susceptible to thermal inactivation at 56°C. To the extent that the composition of intracellular vesicles should be fixed at the time the cells are homogenized, the thermal lability of the acidification response in vesicles prepared from cells grown at 34°C indicates that the heat-sensitive activity is a resident of the acidifying vesicles at 34°C. Thus, the manifestation of the lesion when cells are shifted from 34°C to 39.5°C is not the result of some component that is left out of the vesicles when the temperature is raised.

The acridine orange assay for acidification with crude extracts is nonspecific in the sense that we cannot identify the subcellular organelles from which the acidifying vesicles derive, except to reasonably exclude a mitochondrial origin because the activity was sensitive to NEM and resistant to oligomycin. Thus, the acidifying vesicles could come from the plasma membrane, elements of the endocytic vacuolar system (such as coated vesicles and endosomes), lysosomes, Golgi-derived vesicles, or possibly the endoplasmic reticulum. Since DT, modeccin, and transferrin all apparently utilize a low endosomal pH for their biologic activities, it is likely that the lesion in G.7.1 cells is expressed at least at the level of endosomes. It is interesting that all measurable acidification activity in extracts from G.7.1 cells was essentially lost within ~2 min after heating to 56°C. If vesicles from different subcellular origins contribute to the total acidification activity in the crude extract, then all the vesicles must be affected by the heat-sensitive factor.

We cannot readily explain at present why the mutant cells were far more resistant to modeccin than to DT at 39.5°C. The reason may be related to differences between how modeccin and DT pass from intracellular vesicles to the cytosol. The enzymatic subunit of DT penetrates through a membrane in response to a low pH and the penetration event is initiated from within a prelysosomal vesicle very soon after the toxin is endocytosed (17). The escape of modeccin from vesicles is more complicated. The catalytic subunit of modeccin does not reach the cytosol for over an hour after the toxin has been exposed to a low pH within vesicles and there is evidence that the toxin does not pass through a membrane barrier from early endosomal vacuoles (17, 25). It is likely that modeccin requires other functions of the vacuolar apparatus in addition to a low pH before the A chain can reach the cytosol. A lesion affecting acidification might also interfere with these other functions; therefore, an acidification defect could inhibit more than one step of the pathway taken by modeccin to reach the cytosol and result in greater resistance to modeccin than to DT.

The lesion in mutant G.7.1, because it is induced at elevated temperature, appears to be different than the lesion responsible for the acidification defect in the DPV^r class of mutants (7, 11, 33). These mutants were maintained in Ham's F-12 growth medium, which contains 3 μM FeSO$_4$. It would be interesting to determine if the DPV^r mutants would grow in medium with less iron. The mutant described by Robbins et al. (10), which was defective in several aspects of endocytic function, including the transferrin-mediated acquisition of iron (41), was fully sensitive to the cytotoxic action of PE and modeccin and must be different in some respect from the DPV^r mutants and mutant G.7.1. It is apparent that protein toxins can be valuable tools for isolating mutants that carry different lesions affecting endocytic function. The ability to isolate these mutants in conditional-lethal form expands a genetic approach to studying the molecular mechanisms of endocytosis. For example, by placing mutagenized cultures of G.7.1 at 39.5°C, we have recently isolated revertants that can grow at the elevated temperature. Further study of revertants should prove interesting.

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