Impaired Lysosomes in a Temperature-sensitive Mutant of Chinese Hamster Ovary Cells

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Abstract. We describe here the properties of a mutant of Chinese hamster ovary cells that expresses a conditional-lethal mutation affecting dense lysosomes. This mutant, termed V.24.1, is a member of the End4 complementation group of temperature-sensitive mutants selected for resistance to protein toxins (Colbaugh, P. A., C.-Y. Kao, S.-P. Shia, M. Stookey, and R. K. Draper. 1988. *Somatic Cell Mol. Genet.* 14:499–507). Vesicles present in postnuclear supernatants prepared from V.24.1 cells harvested at the restrictive temperature had a 50% reduction in acidification activity, assessed by the ATP-stimulated accumulation of the dye acridine orange in acidic vesicles. To investigate whether specific populations of vesicles were impaired in acidification, we measured acidification activity in three subcellular fractions prepared from Percoll gradients: one containing endosomal and Golgi markers, one containing buoyant lysosomes, and the third containing dense lysosomes. Activity in dense lysosomes was reduced by 90%, activity in the buoyant lysosome fraction was unaffected, and activity in the endosome–Golgi fraction was mildly reduced. The activity of three lysosomal enzymes—β-hexosaminidase, β-galactosidase, and β-glucocerebrosidase—was also reduced in dense lysosomes but nearly normal in the buoyant lysosome fraction. However, β-hexosaminidase and β-glucocerebrosidase activity was increased two- to threefold in the endosome–Golgi fraction. We conclude that the lesion selectively impairs dense lysosomes but has little effect on properties of buoyant lysosomes.

Lysosomes are the product of two converging pathways of membrane flow. Endogenous lysosomal enzymes (38) and at least some membrane proteins destined for lysosomes (13) are synthesized on the rough endoplasmic reticulum, traverse the Golgi apparatus, and are sorted into a pathway leading to lysosomes. The sorting of soluble lysosomal enzymes is initiated by addition of the mannose-6-phosphate recognition marker in the Golgi apparatus, which is recognized by the mannose-6-phosphate receptor (MPR)[1] (15, 38). MPRs carry the enzymes to an acidic prelysosomal compartment where they dissociate and eventually reach lysosomes (5, 15). Unoccupied MPRs are recycled back to the Golgi apparatus and reused (5). Transport to and from the prelysosomal compartment is believed to be mediated by clathrin-coated vesicles (5, 11, 14). Lysosomes also receive membrane and vesicle contents from the endocytic pathway. Endocytosed substances meet newly synthesized lysosomal enzymes in a prelysosomal compartment that has been difficult to characterize. Griffiths et al. (15) recently described a compartment that is the apparent site of MPR recycling. This compartment shares many features with lysosomes, including an acidic interior, lysosomal enzymes, and possibly degradative activity. Griffiths et al. (15) proposed that this prelysosomal compartment is a specialized endosome that serves as an intermediate in lysosomal biogenesis.

Although a general outline of the endosomal–lysosomal pathway is understood, the specific steps of the pathway remain poorly characterized. For example, the number of discrete vesicle intermediates involved in lysosome biogenesis is not yet clear. Moreover, there appear to be two types of lysosomes, buoyant and dense, both of which contain hydrolytic enzyme activity and can receive endocytosed material (26, 30). The role of buoyant lysosomes in the endocytic pathway is unclear, although it has been suggested that they are precursors of dense lysosomes (3, 21, 26, 30). To address questions about the endosomal–lysosomal pathway, we have sought to isolate temperature-sensitive mutants of mammalian cells defective in the pathway. Recently, we described a temperature-sensitive derivative of Chinese hamster ovary (CHO) cells, termed mutant V.24.1, that defined a fourth complementation group (End4) of CHO cell mutants defective in the endosomal–lysosomal pathway (7). Mutant V.24.1 was selected for resistance to diphtheria toxin at 39.5°C, was cross-resistant to the plant toxin modeccin, and had a reduced ATP-stimulated acidification activity of vesicles in postnuclear supernatants. We report here that the acidification activity and lysosomal enzyme activity associated with dense lysosomes are impaired in the mutant at the restrictive temperature, although these activities appear to be almost normal in subcellular fractions containing buoyant lysosomes.

1. *Abbreviations used in this paper*: CHO, Chinese hamster ovary; MPR, mannose-6-phosphate receptor.

Materials and Methods

Materials

Modeccin was obtained from Pierce Chemical Co. (Rockford, IL). The following materials were obtained for cell culture: Falcon disposable culture plates (Falcon Labware, Oxnard, CA); Nunc disposable culture plates (Southland Cryogenic, Carrolton, TX); Costar disposable culture plates (Costar, Cambridge, MA); FBS (Hazleton Research Products, Denver, PA); powdered growth medium and fungizone (Irvine Scientific, Santa Ana, CA). Ethyl methanesulfonate was purchased from Eastman Kodak Co. (Rochester, NY); methylumbelliferyl substrates for enzyme assays and bovine hemoglobin type I were purchased from Sigma Chemical Co. (St. Louis, MO). Sources for other reagents have been previously identified (7, 18, 35).

Cells

Growth medium and procedures of cell culture were as previously reported (7, 18) with the exception that Hepes was omitted from the growth medium. CHO-K1 was obtained from the American Type Culture Collection (Rockville, MD). The isolation of the temperature-sensitive mutant V.24.1 has been previously reported (7).

The revertant V.24.1R31 was selected from V.24.1 by growth at 39.5°C. V.24.1 cells growing at 34°C were mutagenized with 75 μg/ml ethyl methanesulfonate for 24 h. The ethyl methanesulfonate was removed, the cells were washed with PBS, and fresh growth medium was added to the culture dishes. After overnight incubation at 34°C, the cells were shifted to 39.5°C and surviving cells were allowed to grow for 3 wk. Growth medium was changed every 3–5 d, as needed. Surviving colonies were picked and grown at 34°C. Revertants were obtained at a frequency of 4×10^{-5} in this isolation.

Protein Synthesis Assays

The protein synthesis assay for the activity of toxins on cultured cells has been described (7, 18). Briefly, cells in 24-well Falcon plates containing medium with 100-fold less leucine than normal were exposed to 2 μCi/ml of L-[4,5-^{3}H]leucine for 1 h, washed with PBS, and the radioactivity incorporated into acid-insoluble material was measured. All assays were done in triplicate.

Acidification Assays

The initial rate of acridine orange accumulation within acidic vesicles was measured as the difference between the absorbance at 492 nm and 540 nm with a dual-wavelength spectrophotometer (Aminco-Chance, Urbana, IL) using the method of Stone et al. (34, 35). Cells were harvested by trypsinization and homogenized at 4°C with a Dounce homogenizer (Kontes Co., Vineland, NJ) in a buffer containing 10 mM Hepes, 0.25 M sucrose, 2 mM EDTA, pH 7.4. Postnuclear supernatants were prepared by centrifuging the homogenate at 800 *g* for 10 min. Extracts were mixed with acidification buffer and the reaction was initiated by addition of 1 mM ATP. Acidification buffer consisted of 30 mM histidine, 130 mM NaCl, 20 mM KCl, 2 mM $MgCl_2$, and 2.5 μM acridine orange, pH 7.0. Protein was measured by the bicinchoninic acid method of Smith et al. (33).

Parameters of the Transferrin Cycle

Diferric transferrin was iodinated as previously described (37). Parental and V.24.1 cells were inoculated into 4- or 24-well culture dishes 2 d before the experiments and grown at 34°C. To induce lesion expression, cells were shifted to 39.5°C for the final 16 h. The cells were washed twice with PBS and preincubated with DME, minus serum, containing 1 mg/ml BSA (binding medium) for 1 h before assaying. To measure ^{125}I-transferrin accumulation, the preincubation medium was replaced with 30 nM ^{125}I-transferrin in binding medium at indicated intervals and the cells were incubated at 39.5°C. Plates were then chilled and the wells were washed three times with ice-cold PBS containing 1 mg/ml BSA. Cells were treated with 0.5 mg/ml Pronase in PBS for 10 min at 4°C to remove surface ^{125}I-transferrin, the Pronase was diluted with an equal volume of FCS, and the cells were sedimented at 4°C in an Eppendorf microcentrifuge. The cell pellets were counted in a gamma counter (model 5500; Beckman Instruments, Inc., Fullerton, CA). To measure the recycling of transferrin from cells, cells were allowed to accumulate ^{125}I-transferrin for 30 min at 39.5°C, the plates were chilled and washed as described above, and binding medium at 39.5°C was added to the cells. At intervals, cells were chilled and washed, incubated with Pronase, and the cell-associated radioactivity was determined as described above. All experiments were performed in triplicate. In parallel experiments, a 100-fold excess of unlabeled transferrin was present in addition to 30 nM ^{125}I-transferrin to assess nonspecifically bound radioactivity. Nonspecifically bound radioactivity was subtracted from the total bound radioactivity to yield specific binding. Data is expressed as specific cpm per cell.

Transferrin was loaded with $^{59}Fe^{+3}$ as previously described (37) and had a specific activity of 3×10^3 cpm/μg. To measure iron accumulation, 60-mm culture dishes containing 2–3 $\times$ 10^6 cells/plate were incubated at 39.5°C for 17 h and after a 1-h preincubation in binding medium were exposed to 30 nM transferrin labeled with $^{59}Fe^{+3}$ for different times. Cells were chilled, washed three times with PBS containing 1 mg/ml BSA, solubilized in 1.0 N NaOH, and assayed for radioactivity by gamma counting. Nonspecific uptake was measured in the presence of a 500-fold excess of unlabeled diferric transferrin and was subtracted from total uptake to yield specific iron accumulation.

Enzyme Assays

The activities of the lysosomal enzymes β-hexosaminidase and β-galactosidase in subcellular fractions were assayed fluorometrically using the 4-methylumbelliferyl substrates as described by Robbins (27). β-Glucocerebrosidase was assayed by a modification of the method of Mueller and Rosenberg (23). 150 μl of sample was incubated with 250 μl of 0.1 M sodium acetate buffer, pH 4.4, containing 1 mM 4-methylumbelliferyl-β-D-glucoside, 0.1% Triton X-100, and 0.1% sodium taurocholate, at 37°C for 2 h. The reaction was terminated by adding 1 ml of 0.1 M NaOH, and the fluorescence was measured with excitation at 390 nm and emission at 475 nm.

Percoll Gradients

Postnuclear supernatants were fractionated in 17 or 27% Percoll gradients prepared in buffer containing 0.25 M sucrose, 2 mM EDTA, and 10 mM Hepes, pH 7.4. Gradients were prepared by placing 6 ml of cell extract over 28 ml Percoll in quick-seal polyallomer tubes with a 4-ml cushion of 60% wt/vol aqueous sucrose at the bottom. Centrifugation was at 25,000 *g* for 1 h at 4°C in a rotor (VTi 50; Beckman Instruments, Inc.). After centrifugation, fractions of 1.2 ml were collected.

Roff et al. (29) have shown that centrifugation of postnuclear supernatants from CHO cells with 17% Percoll separates vesicles of the endosomal–lysosomal system into two fractions. The buoyant fraction contains a population of endosomes and also contains markers for the Golgi apparatus. The dense fraction from 17% gradients contains unresolved buoyant and dense lysosomes. In a 27% gradient, however, the buoyant lysosomes separate from dense lysosomes and sediment with endosomes and Golgi elements. To quantitate both acidification activity and the activities of lysosomal enzymes in fractions containing endosomes and Golgi markers (low-density fraction), buoyant lysosomes (intermediate-density fraction), and dense lysosomes (high-density fraction), we centrifuged half of a postnuclear supernatant with 27% Percoll and the other half with 17% Percoll. Activity in the endosome–Golgi fraction was obtained from the buoyant peak of the 17% gradient (typically, fractions 10–21). Activity of the dense lysosomes was obtained from the high-density peak of 27% gradients (typically, fractions 22–30). Activity of the buoyant lysosome fraction was obtained by subtracting activity in the buoyant peak of the 17% gradient from the activity in the buoyant peak of the 27% gradient. Alternatively, activity of the buoyant lysosome fraction could be calculated by subtracting the activity of dense lysosomes in the 27% gradient from the high-density peak of the 17% gradient. Both methods for quantitating activities in the buoyant lysosome fraction gave nearly identical results.

Results

Revertants of V.24.1

Mutant V.24.1 carries a temperature-sensitive defect that kills cells at 39.5°C (7). Revertants of V.24.1 cells were selected by placing cultures at 39.5°C for 3 wk. In two separate experiments, spontaneous revertants appeared at a frequency of 7–8 $\times$ 10^{-6}.

One characteristic of V.24.1 cells at elevated temperature

Table I. Sensitivity of Parental, Mutant, and Revertant Cells to Modeccin at 41°C

Cell	IC50	Relative resistance
	nM	
CHO-K1	0.04	1.0
V.24.1	>100	>2,500
V.24.1R31	0.07	1.8

2 d before the experiment 5×10^4 cells were plated at 34°C in 24-well culture dishes. Dishes were shifted to 41°C for 3 h, and the medium was removed and replaced with fresh growth medium containing 1:100 the normal amount of leucine. Varying concentrations of modeccin were then added, and the cells were incubated an additional 3 h at 41°C. L-[4,5-^{3}H]Leucine was added to the growth medium during the last hour of incubation to assess protein synthesis, as described in Materials and Methods. The IC50 is the amount of toxin required to inhibit protein synthesis by 50% compared with controls that did not receive toxin.

Table II. Time to Acquire Modeccin Resistance for V.24.1 Cells at 39.5°C

Time at 39.5°C	IC50 V.24.1	IC50 CHO-K1
h	*nM*	*nM*
0	0.4	0.3
6	0.1	0.03
9	2.0	–
15	1.8	–
24	–	0.1

Cells were plated into 24-well culture dishes, grown at 34°C for 2 d, and then shifted to 39.5°C for the indicated times. Modeccin at various concentrations was added 3 h before the end of the time period; L-[4,5-^{3}H]Leucine was present during the final hour to assess protein synthesis.

is an increase in resistance to the protein toxin modeccin (7). Modeccin is normally internalized by receptor-mediated endocytosis and subsequently penetrates to the cytoplasm from an intracellular vesicle, possibly lysosomes (9, 32), where it catalytically inactivates protein synthesis (10, 25). We compared the sensitivities of wild-type cells, V.24.1 cells, and a revertant, termed V.24.1R31, to modeccin at a restrictive temperature of 41°C (Table I). The revertant had a normal sensitivity to modeccin. In all, 17 revertants, obtained from untreated or mutagen-treated V.24.1 cells, have been assayed for modeccin sensitivity. All revertants tested had a normal sensitivity to modeccin (data not shown), indicating that reversion of the V.24.1 phenotype to survival at the restrictive temperature was tightly correlated with reversion to normal modeccin sensitivity.

Onset of Temperature-sensitive Traits of V.24.1 Cells

We determined the time at nonpermissive temperatures required to elicit temperature-sensitive responses in V.24.1 cells. The time required for V.24.1 cells to lose viability at 39.5°C is shown in Fig. 1. Cells at 34°C were placed at 39.5°C for the times indicated and then returned to 34°C to allow colony formation. Half of the V.24.1 cells were killed after a 25-h exposure to the elevated temperature. In similar experiments where the cells were shifted for varying periods of time to 41°C, rather than 39.5°C, and then returned to 34°C, V.24.1 cells lost viability ~8 h after being shifted to 41°C (data not shown). To determine whether protein synthesis was inhibited in V.24.1 at the elevated temperatures, the rate of L-[4,5-^{3}H]leucine incorporation was measured at 6-h intervals up to 24 h after the cells were shifted to 39.5°C, or after 4 or 8 h of growth at 41°C. Little difference in L-[4,5-^{3}H]leucine incorporation was observed between the mutant and parental cells at either temperature, indicating that V.24.1 did not lose viability at the elevated temperatures because of a general block in protein synthesis (data not shown).

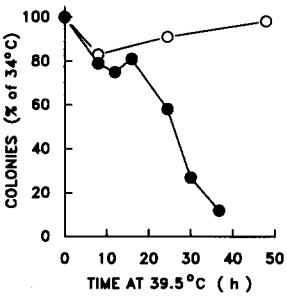

Figure 1. Survival of V.24.1 cells at 39.5°C. Parental and mutant cells were inoculated at 150 cells per culture flask (25-cm^2 surface area) containing growth medium and were allowed to attach overnight at 34°C. Flasks were shifted to 39.5°C for the indicated times and then returned to 34°C for 3 wk. Colonies were stained with 0.5% crystal violet and counted. Values are the average of triplicate assays at each time point and are expressed as the number of colonies on plates shifted to 39.5°C compared with plates that remained at 34°C. (○) CHO-K1; (●) V.24.1.

The time at 39.5°C for cells to acquire resistance to modeccin was determined in experiments where the cells were shifted to the elevated temperature for increasing intervals and then assayed for toxin sensitivity. The data, presented in Table II, indicates that the cells became resistant to modeccin between 6 and 9 h at 39.5°C. Comparison of the modeccin sensitivity of V.24.1 cells at 39.5°C in Table II (and in previous work; reference 7) with that shown in Table I when cells were incubated at 41°C indicates that the slight increase in the restrictive temperature markedly enhanced the resistance of the cells to modeccin. In other experiments using 41°C as the nonpermissive temperature, full resistance to modeccin developed between 5 and 6 h at 41°C (data not shown).

To see whether cells at 41°C could regain normal sensitivity to modeccin after being returned to 34°C and to determine if protein synthesis was required for recovery, the sensitivity to modeccin was assessed in cells placed first at 41°C for 5 h to induce toxin resistance, followed by an 11-h incubation at 34°C in the presence or absence of cycloheximide. V.24.1 cells regained almost full sensitivity to modeccin after a recovery period of 11 h at 34°C, and further, protein synthesis by the cells during this 11-h recovery period was necessary to regain sensitivity (Table III). This result is consistent with the idea that V.24.1 cells express a temperature-sensitive protein that is inactivated at the nonpermissive temperature and which must be resynthesized to restore function at the permissive temperature.

We previously reported that ATP-stimulated acidification activity of vesicles present in postnuclear supernatants prepared from V.24.1 cells harvested after incubation at 39.5°C was reduced relative to the parent CHO-K1 cells (7). To assess the time course for this loss in acidification activity, cells growing at 34°C were shifted to 39.5°C for varying times and the specific ATP-stimulated acidification activity

Table III. Acquisition of Modeccin Sensitivity upon Shifting V.24.1 Cells from 41 to 34°C in the Presence or Absence of Cycloheximide

Condition	IC50	
	Parental	V.24.1
	nM	*nM*
34°C	0.5	1.0
41°C	0.04	>100
34°C (11-h recovery, no cycloheximide)	0.9	1.1
34°C (11-h recovery, with cycloheximide)	2.0	>100

Parental and V.24.1 cells were plated in 24-well culture dishes, four dishes per each cell type, and grown for 2 d at 34°C. Three plates of each cell type were then shifted to 41°C. After 2 h, different concentrations of modeccin were added to cells on one plate of each cell type for 3 h to assess the IC50 for the toxin. The remaining two plates of each cell type were returned to 34°C, with 5 μM cycloheximide added to one of the plates. After 8 h at 34°C, modeccin was added for 3 h to both sets of plates, and to the control plate that had been maintained at 34°C. For all assays, L-[4,5-^{3}H]leucine was added during the last hour of exposure to modeccin. Cycloheximide was removed from cultures during the time L-[4,5-^{3}H]leucine was present.

was measured in postnuclear supernatants. As shown in Table IV, V.24.1 postnuclear supernatants had ~80% of parental acidification activity at 34°C before being shifted to the elevated temperature. After the cells were transferred to 39.5°C, loss of ATP-stimulated acidification activity to approximately half of the wild-type level was observed; this effect was manifested within 6 h.

The Transferrin Cycle Appears Normal in V.24.1 Cells

To see whether the coated pit pathway of endocytosis was operating in V.24.1 cells at high temperature, we compared the rate of transferrin accumulation in mutant cells with parental cells that were incubated at 39.5°C for 16 h. There was no difference between mutant and normal cells (Fig. 2 *A*). We also measured the rate of transferrin recycling from cells preloaded for 30 min with ^{125}I-transferrin. The lesion in V.24.1 cells had no apparent effect on the rate of transferrin loss from cells, as seen in Fig. 2 *B*. Transferrin-mediated uptake of iron by the mutant cells was also not affected by the lesion (Table V). These data indicate that the transferrin cycle is normal in the mutant cells after 16 h at 39.5°C, a time at which modeccin resistance and impaired vesicle acidification are fully expressed.

Table IV. Specific ATP-stimulated Acidification Activity of Postnuclear Supernatants from V.24.1 and Parental Cells as a Function of Time at 39.5°C

Time at 39.5°C	($dA_{492-540}$ mg^{-1} ml^{-1}) × 10^2		Relative acidification activity
	CHO-K1	V.24.1	
h			
0	3.25 ± 0.22	2.54 ± 0.08	0.78 ± 0.06
6	3.23 ± 0.34	1.43 ± 0.06	0.44 ± 0.05
12	3.27 ± 0.23	1.50 ± 0.41	0.46 ± 0.13
18	3.06 ± 0.20	1.43 ± 0.12	0.47 ± 0.05
24	3.85 ± 0.40	2.06 ± 0.09	0.54 ± 0.06

Cells were plated at 34°C and allowed to grow until nearly confluent. The plates were shifted to 39.5°C for the indicated times, and postnuclear supernatants were prepared. ATP-stimulated acidification activity was assayed as described in Materials and Methods. Standard deviations from the mean of triplicate measurements are indicated.

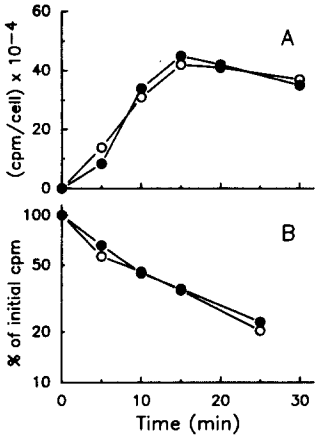

Figure 2. Accumulation and efflux of ^{125}I-transferrin by mutant and parental cells. Accumulation and efflux of transferrin by parental and mutant cells was measured at 39.5°C as described in Materials and Methods. *A* shows the accumulation of transferrin as a function of time of incubation with the radioactive ligand. *B* shows the loss of cell-associated ^{125}I-transferrin from cells after a 30-min accumulation period. (○) CHO-K1; (●) V.24.1.

CHO mutants with defective endosomal ATP-stimulated acidification activity internalize transferrin normally, but are unable to extract iron from diferric transferrin as a result of the elevated pH of the endosomes (17, 29, 37). We previously demonstrated that the temperature-sensitive mutant G.7.1, which is defective in endosomal acidification, could grow at 39.5°C if supplemental iron was added to the growth medium (37). We therefore investigated whether supplemental iron in the growth medium could also rescue V.24.1 cells at the restrictive temperature. Growth of parental and V.24.1 cells at 39.5°C was compared when the cells were grown in DME alone or in DME supplemented with either $FeSO_4$ or hemoglobin. The results are summarized in Table VI. The growth of parental cells was unaffected by either form of supplemental iron at the concentrations added. Although both supplements fully rescued mutant G.7.1, which was included in this experiment as a control, neither rescued V.24.1 cells; at 39.5°C both untreated and iron-treated V.24.1 cells rounded and detached from the dishes after a single doubling. This confirms that some factor other than iron starvation is responsible for the lethality of the defect, consistent with the observation that the transferrin cycle appears normal in the mutant cells.

The Defect in V.24.1 Affects Lysosomal Acidification

In the experiment presented in Table IV, postnuclear supernatants were used to detect the loss of ATP-stimulated acid-

Table V. Iron Accumulation in Mutant and Wild-Type Cells at 39.5°C

Time	Cell-associated ^{59}Fe	
	CHO-K1	V.24.1
min	*cpm per 10^6 cells*	*cpm per 10^6 cells*
0	0	4
30	27	25
60	48	58
90	73	75
120	96	110

Table VI. The Effect of Supplemental Iron on Growth of V.24.1 Cells at 39.5°C

Cell	Supplement	Number of doublings
CHO-K1	None	4.31
	$FeSO_4$	4.32
	Hemoglobin	4.29
G.7.1	None	0.68
	$FeSO_4$	3.61
	Hemoglobin	3.22
V.24.1	None	0.15
	$FeSO_4$	0.30
	Hemoglobin	1.30

Cells were plated in DME at a density of 10^4 cells per well in 24-well culture dishes. After overnight incubation at 34°C, cells were counted and either 1.5 μM $FeSO_4$ or 100 μg/ml dialyzed hemoglobin was added to the wells. The cells were then grown at 39.5°C for 72 h, counted, and the number of doublings calculated. Presented are the mean of triplicate assays.

ification activity in V.24.1 cells. However, these crude extracts provided no information as to what vesicles within the cells manifest this defect. To further characterize the reduction of acidification activity, postnuclear supernatants from V.24.1 and parental cells grown at 39.5°C were fractionated on Percoll gradients and the ATP-stimulated partitioning of acridine orange into vesicles was measured for each fraction. The distribution of acidification activity in 27% Percoll gradients is shown in Fig. 3, *A* and *B*. Activity from parental cells was distributed almost equally between dense lysosomes and the more buoyant fraction that is known to contain markers for endosomes, Golgi, and buoyant lysosomes (29). In contrast, almost 90% of the total acidification activity of V.24.1 cells was associated with the more buoyant fraction. The distribution of acidification activity in a 17% Percoll

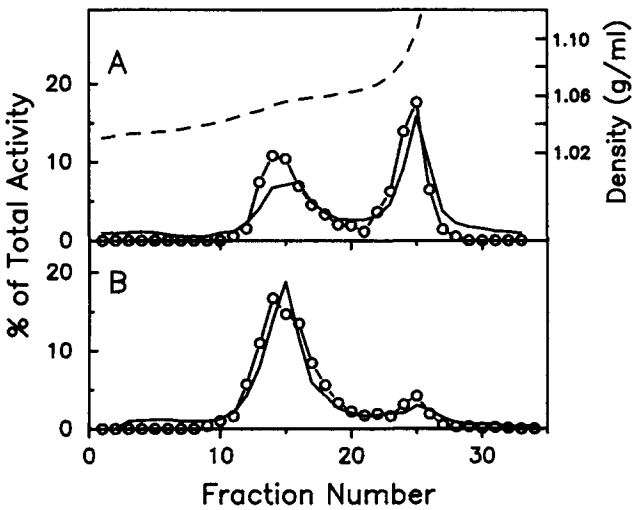

Figure 3. Distribution of ATP-stimulated acidification activity in 27% Percoll gradients. Parental (*A*) or mutant (*B*) cells were harvested after 17 h at 39.5°C; postnuclear supernatants were prepared and centrifuged with 27% Percoll. Each fraction was assayed for ATP-stimulated acidification activity (○) and for β-hexosaminidase activity (———). Data is plotted as percent of total activity. Density is indicated in *A* (- - - -).

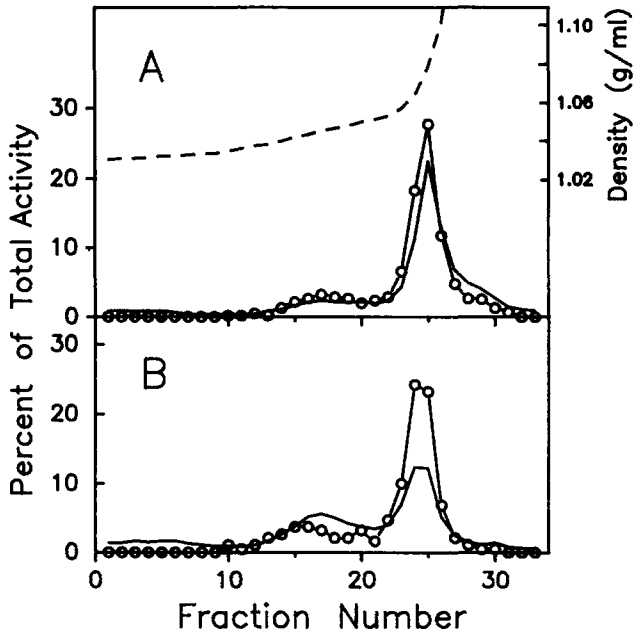

Figure 4. Distribution of ATP-stimulated acidification activity in 17% Percoll gradients. Postnuclear supernatants from parental (*A*) and V.24.1 (*B*) cells grown at 39.5°C for 17 h were centrifuged with 17% Percoll, and the ATP-stimulated acidification activity of each fraction was measured (○). The β-hexosaminidase activity for each fraction is also indicated (———). Density is shown in *A* (- - - -).

gradient is compared for mutant and parental cells in Fig. 4. For parental cells, ~20% of the total activity was associated with the low-density region that contains endosomes and elements of the Golgi apparatus, while 80% was in the high-density region that contains buoyant and dense lysosomes. The relative distribution of acidification activity in the two regions for V.24.1 cells was similar to that for parental cells.

As Roff et al. (29) showed, centrifugation of postnuclear supernatants of CHO cells with both 17 and 27% Percoll is sufficient to define three fractions of the endosomal–lysosomal apparatus. The low-density fraction observed on 17% Percoll gradients contains a population of endosomes, as well as Golgi markers. The high-density fraction from a 27% gradient contains dense lysosomes, while buoyant lysosomes sediment with the high-density fraction in 17% Percoll and with the low-density fraction in 27% Percoll. In Fig. 5 we combined data from the 17 and 27% gradients to compare mutant and parental acidification activity associated with low-density vesicles (*I*), intermediate-density vesicles (*II*), and high-density vesicles (*III*). The single-lined bars represent the distribution for the mutant relative to the total activity recovered. To normalize the comparison with respect to protein originally present in the postnuclear supernatants, the double-lined portion of the bars is 47% of the single-lined bars, which accounts for the 53% reduction in specific acidification activity in extracts from the mutant cells after 18 h at 39.5°C (see Table IV). Comparison of the adjusted distribution for the mutant with the distribution for the parental cells indicates that the acidification activity in the low-density fraction of the mutant is somewhat reduced while activity in the intermediate-density fraction for the mutant is

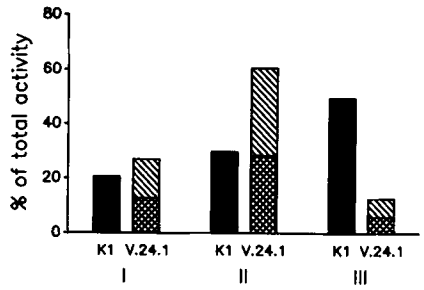

Figure 5. Comparison of parental and mutant acidification activity sedimenting with vesicles of low, intermediate, and high buoyant density. Parental CHO-K1 (*solid bars*) and V.24.1 (*lined bars*) cells grown at 39.5°C for 17 h were centrifuged with 17 and 27% Percoll. The acidification activity was measured for each fraction (shown in Figs. 3 and 4), and the percent of total activity in vesicles of low (*I*), intermediate (*II*), and high (*III*) density was determined as described in Materials and Methods. The relative acidification activity of the mutant compared with parental cells (calculated by multiplying the percent of total activity for each population of vesicles of the mutant by 0.47 [See Table IV], the factor by which total acidification activity for the mutant is reduced compared with parental cells after 18 h at 39.5°C) is indicated by double-lined bars.

nearly identical to that of normal cells. The major difference, however, is a 90% loss of acidification activity in the high-density fraction of the mutant that should contain dense lysosomes. Thus, either the acidification capacity of dense lysosomes in V.24.1 cells is severely impaired or dense lysosomes are not present.

Localization of Lysosomal Enzymes in V.24.1 Cells Is Altered

Also shown in Figs. 3 and 4 is the sedimentation of β-hexosaminidase activity in 17 and 27% Percoll gradients for mutant and parental cells. As was seen with acidification activity, the relative amount of β-hexosaminidase activity associated with the dense lysosomal peak (27% Percoll gradients; Fig. 3) in V.24.1 cells grown at 39.5°C was reduced compared with parental cells. In 17% Percoll gradients (Fig. 4), the β-hexosaminidase activity in the buoyant peak from the mutant cells appeared to be somewhat increased compared with the parental cells. To further investigate the distribution of lysosomal enzymes in subcellular compartments, we compared the sedimentation of β-hexosaminidase, β-galactosidase, and β-glucocerebrosidase activity in fractionated postnuclear supernatants from parental and mutant cells. β-Hexosaminidase and β-galactosidase depend on the MPR for sorting in the Golgi apparatus and transport to lysosomes, whereas β-glucocerebrosidase, a membrane-associated enzyme, is sorted to lysosomes independent of the MPR (1, 36). The results are summarized in Table VII. The specific activity of each enzyme was assessed in postnuclear supernatants before centrifugation with 17 and 27% Percoll. There was slightly more β-hexosaminidase and β-glucocerebrosidase activity in the mutant cells at the restrictive temperature, a result opposite to that observed with acidification activity. β-Galactosidase activity, however, was reduced to about half the parental level. To compare the distribution of lysosomal enzymes among fractions for mutant and parental cells, the percent of the total activity associated with low- (*I*), intermediate- (*II*), and high- (*III*) density fractions from the mutant was multiplied by 1.28 for β-hexosaminidase, 0.57 for β-galactosidase, and 1.18 for β-glucocerebrosidase, and divided by the activity found for wild-type cells to give the relative activity in each of the three fractions. As seen in Table VII, there was 50, 70, and 30% less activity associated with dense lysosomes from V.24.1 cells for β-hexosaminidase, β-galactosidase, and β-glucocerebrosidase, respectively. There was three times as much β-hexosaminidase and almost two times as much β-glucocerebrosidase in low-density fractions of V.24.1 cells, but the activity of β-galactosidase was not increased in low-density fractions. Enzyme activity in fractions of intermediate density were not much different between mutant and parental cells.

Acidification and β-Hexosaminidase Activities in V.24.1 Cells at 34°C and in a Revertant at 39.5°C

To determine whether the loss of acidification and lysosomal enzyme activity from dense lysosomes of V.24.1 cells resulted from incubation at the restrictive temperature, a postnuclear supernatant was prepared from cells harvested at 34°C and centrifuged with 27% Percoll. The buoyant and dense peaks were collected and assayed for β-hexosaminidase and acidification activities (Table VIII). The dense peak, which normally contains ~50% of both activities for wild-type cells in 27% gradients, had 34 and 36% of the total β-hexosaminidase and acidification activity, respectively, for V.24.1 cells. Thus, there is a mild reduction in these activities associated with dense lysosomes from the mutant cells at the permissive temperature. This data is consistent with the previous observation that the total acidification activity in V.24.1 cells at 34°C is reduced by ~20% (see

Table VII. Comparison of Lysosomal Enzyme Activity in Fractionated Postnuclear Supernatants from V.24.1 and Parental Cells Grown at 39.5°C

Enzyme	Cell	Relative specific activity in postnuclear supernatants	Subcellular distribution: Total activity I	II	III	Relative activity I	II	III
			%	%	%			
β-Hexosaminidase								
	CHO-K1	1.00	22	26	52	1.0	1.0	1.0
	V.24.1	1.28	51	29	20	3.0	1.4	0.5
β-Galactosidase								
	CHO-K1	1.00	13	20	67	1.0	1.0	1.0
	V.24.1	0.57	23	43	34	1.0	1.2	0.3
β-Glucocerebrosidase								
	CHO-K1	1.00	35	20	45	1.0	1.0	1.0
	V.24.1	1.18	54	18	28	1.8	1.1	0.7

Cells were grown at 39.5°C for 16 h, trypsinized, and postnuclear supernatants prepared. The specific activity of each enzyme in the postnuclear supernatants was determined and is presented relative to parental values. Postnuclear supernatants were centrifuged with 17 and 27% Percoll, and enzyme activities were measured in each fraction. The percent of sedimentable activity associated with vesicles of low (I), intermediate (II), or high density (III) was calculated as described in Materials and Methods. Protein content of the postnuclear supernatants was determined by the method of Bradford (4).

Table VIII. Distribution of Acidification and β-Hexosaminidase Activities between Buoyant and Dense Peaks in 27% Percoll for V.24.1 Cells at 34°C and a Revertant at 39.5°C

Cell	Activity	Total activity	
		Buoyant peak	Dense peak
		%	%
V.24.1 (34°C)	Acidification	66	34
	β-Hexosaminidase	64	36
V.24.1R31 (39.5°C)	Acidification	56	44
	β-Hexosaminidase	47	53

Table IV). A similar experiment was done using the revertant V.24.1R31 incubated at 39.5°C before harvesting the cells (Table VIII). The distribution of acidification and β-hexosaminidase activities between the bouyant and dense peaks was nearly identical to that for wild-type cells, indicating that the mutant phenotype was returned to normal in the revertant.

Discussion

Efforts to isolate mutants of mammalian cells that express temperature-sensitive lesions affecting the endosomal–lysosomal pathway have so far produced mutants defining five complementation groups (7). Mutants representing the End1, End2, and End3 complementation groups are defective in endosomal acidification (18, 22, 28, 29, 31, 37). A mutant of the fifth complementation group, End5, has not yet been studied in detail (7). We have described here some properties of mutant V.24.1, which represents the End4 complementation group. At the restrictive temperature, multiple phenotypic changes occur in V.24.1 cells, including failure to grow, resistance to modeccin and diphtheria toxin, a reduction in ATP-stimulated acidification of subcellular vesicles, and alterations in lysosomal enzyme activity. All of these traits return toward normal in revertants of V.24.1 cells, suggesting that all are the consequence of a single genetic lesion. Also, recovery of V.24.1 cells to modeccin sensitivity at 34°C after incubation at the nonpermissive temperature was inhibited by cycloheximide, consistent with the idea that the temperature-sensitive properties of the mutant result from inactivation of a heat-labile protein that must be resynthesized to enable recovery.

The earliest phenotypic change observed for V.24.1 cells after a temperature shift to 39.5°C is loss of ATP-stimulated acidification activity, which is fully manifested within 6 h after the shift. Another trait expressed at approximately the same time is resistance to modeccin. Loss of cell viability at the nonpermissive temperature occurs ~12 h after the expression of toxin resistance and loss of acidification activity. The cause of cell death at 39.5°C has not been determined, but apparently is not due to the absence of protein synthesis or to iron starvation, since mutant cells incorporate L-[4,5-^{3}H]leucine at parental levels before they die and are not rescued by supplemental iron in the medium.

At 39.5°C, the total acidification activity in postnuclear supernatants of V.24.1 cells was down by ~50% and most of this loss was accounted for by the absence of activity normally associated with the region in Percoll gradients where dense lysosomes sediment. We conclude from this that dense lysosomes are either defective in acidification or they are not present. The data is not consistent with an interpretation where only the buoyant density of lysosomes has been altered so that they appear in another region of the gradient. If this were true, there should not have been a reduction in total acidification activity and we should have observed an increase in activity at a new location in Percoll gradients. We do not, however, rule out the possibility that both the density and acidification activity of dense lysosomes might be altered in the mutant.

The lesions in mutants of the End1, End2, and End3 classes impair endosome acidification, but there is little influence on dense lysosome acidification (22, 28, 29, 31, 37). For V.24.1 cells, dense lysosomes were affected, but there was no significant effect on the buoyant lysosome fraction, and only a partial reduction in the acidification of the endosome–Golgi fraction. Since both endosomes and the *trans*-Golgi are acidifying organelles (2, 12, 20), we cannot tell from the acridine orange assay whether the mild reduction in the endosome–Golgi fraction affects one or both of these organelles. However, since transferrin-mediated iron uptake was normal in the mutant cells and this process requires a low endosomal pH, the endosomal pH in V.24.1 cells is at least low enough to remove iron from transferrin. It is apparent that the acidification lesion in V.24.1 cells has properties very different than those of the other CHO cell mutants. Recently, Cain and Murphy (6) described a chloroquine-resistant derivative of mouse 3T3 cells, CHL60-64, that had reduced acidification of lysosomes. Genetic complementation analysis will be necessary to establish whether the lesions in mutants V.24.1 and CHL60-64 are distinct.

For V.24.1 cells at 39.5°C, the activity of two soluble lysosomal enzymes, β-hexosaminidase and β-galactosidase, was reduced by 50 and 70% in dense lysosomes, respectively, and there was a 30% reduction in the activity of the membrane-associated enzyme β-glucocerebrosidase. The total activity of β-hexosaminidase and β-glucocerebrosidase, however, was not reduced. This suggests that these two enzymes are not being inactivated within dense lysosomes and also suggests that they are not being secreted from the cells, although we have not directly measured secretion. We cannot, however, rule out the possibility that the enzymes are inactivated or secreted and that there is a compensating increase in biosynthesis to maintain normal enzyme levels. It is also interesting that the aberrant distribution of both β-hexosaminidase and β-glucocerebrosidase in V.24.1 cells is so similar considering that β-hexosaminidase is sorted to lysosomes via the MPR pathway and β-glucocerebrosidase is not (1, 36, 38). Thus, the lesion in the mutant cells is not specific to one sorting mechanism while leaving an alternative mechanism unaffected. Although the activity of β-galactosidase associated with dense lysosomes was down by ~70% in V.24.1 cells at elevated temperature, the total activity of β-galactosidase in V.24.1 cells was also reduced ~50%, unlike results with β-hexosaminidase and β-glucocerebrosidase. The reason for this difference is not apparent at present.

The decline in β-hexosaminidase and β-glucocerebrosidase activity associated with dense lysosomes of V.24.1 cells at 39.5°C was accompanied by an increase in the activity of

both enzymes in low-density vesicles. One explanation for this is that dense lysosomes become buoyant at 39.5°C, carrying with them their complement of enzymes. If this occurs, however, then the lysosomes whose density is shifted must also lose the ability to acidify since the acidification activity of buoyant vesicles was not increased. An alternative possibility is that β-hexosaminidase and β-glucocerebrosidase are accumulating in a prelysosomal compartment, possibly the Golgi apparatus, or a subsequent vesicle en route to dense lysosomes. Further work will be necessary to resolve these possibilities. Nevertheless, lysosomal enzymes of V.24.1 cells retain latency at the high temperature, as indicated by the absence of soluble activity at the top of Percoll gradients. Thus, lysosomes of the mutant cells are not bursting and the lethality of the lesion is apparently not the result of lysosomal enzymes released into the cytoplasm.

The acidification and lysosomal enzyme activities in the buoyant lysosome fraction were nearly normal in the mutant at the restrictive temperature, suggesting that the lesion discriminates between buoyant and dense lysosomes. Domsch and Mersmann (8) suggested that resolution of lysosomes into buoyant and dense species represents an artifact of the shape of self-forming Percoll gradients, and that only a single population is actually present. Mutant V.24.1, however, provides genetic evidence that the two populations of lysosomal vesicles are physically distinct.

While the lesion in V.24.1 cells impairs dense lysosomes, early aspects of the endocytic pathway appear unaffected, as indicated by the normal uptake and recycling of transferrin. We previously noted that V.24.1 cells accumulated less radiolabeled transferrin than normal cells in a rapid screening test for transferrin uptake at the restrictive temperature (7), a result different than observed here. However, in our earlier work, cells were incubated at 39.5°C for 30 h before assessing transferrin uptake. We now know, based on studies presented here, that only ~25% of the cells remain viable after 30 h at 39.5°C. This suggests that the reduced transferrin uptake previously observed was a late consequence of the lesion, probably related to severely stressing the cells upon prolonged incubation at the high temperature.

The resistance of V.24.1 cells to modeccin and diphtheria toxin at 39.5°C could arise in several ways. One possibility is that the lesion impairs the transport of cell surface receptors from the Golgi region to the plasma membrane, causing toxin resistance by reducing the number of toxin receptors. A second is that the acidification of endosomes in the mutant cells is mildly defective, although this seems unlikely considering that iron uptake by the mutant was normal. Nevertheless, the possibility of a mild defect in endosomal acidification of V.24.1 cells is compatible with the slight reduction in the accumulation of acridine orange in buoyant vesicles of the mutant cells (Fig. 5). A third explanation is that the defective lysosomes upset vesicular traffic through late endosomal vesicles. This could impair the action of modeccin which appears to penetrate to the cytoplasm from a late compartment (9, 32). Also, diphtheria toxin does not begin arresting protein synthesis in cells until after a minimum lag period of 15–20 min, consistent with the possibility that this toxin may also need to reach a late endosome before entering the cytoplasm (16, 19).

In conclusion, we have described here the initial characterization of a unique temperature-sensitive mutant of CHO cells that has defective dense lysosomes but whose buoyant lysosomes are apparently normal. The complex phenotype of the mutant appears to result from a single and revertible genetic lesion. Although the primary defect has not yet been identified, V.24.1 cells provide an interesting model system for future studies of lysosome biology.

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