Acidification of Endosome Subpopulations in Wild-Type Chinese Hamster Ovary Cells and Temperature-sensitive Acidification-defective Mutants

Sandra Schmid,* Renate Fuchs,* Margaret Kielian,‡ Ari Helenius,* and Ira Mellman*

*Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510; and
‡Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461

Abstract. During endocytosis in Chinese hamster ovary (CHO) cells, Semliki Forest virus (SFV) passes through two distinct subpopulations of endosomes before reaching lysosomes. One subpopulation, defined by cell fractionation using free flow electrophoresis as "early endosomes," constitutes the major site of membrane and receptor recycling; while "late endosomes," an electrophoretically distinct endosome subpopulation, are involved in the delivery of endosomal content to lysosomes. In this paper, the pH-sensitive conformational changes of the SFV E1 spike glycoprotein were used to study the acidification of these defined endosome subpopulations in intact wild-type and acidification-defective CHO cells. Different virus strains were used to measure the kinetics at which internalized SFV was delivered to endosomes of pH $\leq$6.2 (the pH at which wild-type E1 becomes resistant to trypsin digestion) vs. endosomes of pH $\leq$5.3 (the threshold pH for E1 of the SFV mutant *fus-1*). By correlating the kinetics of acquisition of E1 trypsin resistance with the transfer of SFV among distinct endosome subpopulations defined by cell fractionation, we found that after a brief residence in vesicles of relatively neutral pH, internalized virus encountered pH $\leq$6.2 in early endosomes with a $t_{1/2}$ of 5 min. Although a fraction of the virus reached a pH of $\leq$5.3 in early endosomes, most *fus-1* SFV did not exhibit the acid-induced conformational change until arrival in late endosomes ($t_{1/2}$ = 8–10 min). Thus, acidification of both endosome subpopulations was heterogeneous. However, passage of SFV through a less acidic early endosome subpopulation always preceded arrival in the more acidic late endosome subpopulation. In mutant CHO cells with temperature-sensitive defects in endosome acidification in vitro, acidification of both early and late endosomes was found to be impaired at the restrictive temperature (41°C). The acidification defect was also found to be partially penetrant at the permissive temperature, resulting in the inability of any early endosomes in these cells to attain pH $\leq$5.3. In vitro studies of endosomes isolated from mutant cells suggested that the acidification defect is most likely in the proton pump itself. In one mutant, this defect resulted in increased sensitivity of the electrogenic H^+ pump to fluctuations in the endosomal membrane potential.

We have previously shown, using cell fractionation by free flow electrophoresis (FFE)[1] that internalized Semliki Forest virus (SFV) passes sequentially through two physically distinct subpopulations of endosomes en route to lysosomes in Chinese hamster ovary (CHO) cells (Schmid et al., 1988). Internalized SFV first appears ($t_{1/2}$ = 3–5 min) in an anodally deflected endosomal compartment of relatively low electrophoretic mobility, defined as "early" endosomes. After an ~2 min lag, SFV next appears ($t_{1/2}$ = ~8 min) in an endosomal fraction, defined as "late" endosomes, which migrates more anodally following FFE (Schmid et al., 1988). These two endosome subpopulations have been characterized and found to be biochemically and functionally distinct by a number of criteria (Schmid et al., 1988): (*a*) they have different but overlapping protein compositions; (*b*) early endosomes appear to be the major site of membrane and receptor recycling while late endosomes are involved in delivery of the endosomal content to lysosomes; (*c*) both early and late endosomes contain an ATP-dependent proton pump, however, early endosome acidification in vitro occurs at a slower rate and to a lesser extent than for late endosomes; and (*d*) early (but not late) endosomes contain a functional Na^+, K^+-ATPase activity which limits their capacity for ATP-dependent acidification in vitro, possibly by helping to

Dr. S. Schmid's current address is Research Institute of Scripps Clinic, 10666 N. Torrey Pines Rd., La Jolla, California 92037.

1. *Abbreviations used in this paper*: BHK, baby hamster kidney; CHO, Chinese hamster ovary; FFE, free flow electrophoresis; SFV, Semliki Forest virus; Tfn, transferrin.

establish an interior-positive membrane potential which opposes the activity of the proton pump (Fuchs et al., 1989*a*).

The regulation of endosomal pH is important since the site (or time after internalization) at which a particular acid-sensitive receptor-ligand complex dissociates may influence the efficiency or pathway of subsequent receptors recycling (Helenius et al., 1983; Mellman et al., 1986; Davis et al., 1987). Studies of endocytosis in intact cells have shown that internalized macromolecules encounter compartments of progressively increasing acidity en route to lysosomes (Merion et al., 1983; Murphy et al., 1984; Kielian et al., 1986; Yamashiro and Maxfield, 1987). However, it remains unclear whether this progressive acidification represents a pH drop in individual endosomes or transfer of endocytic tracers from one endosome population with fixed pH to another with a lower average pH.

In this paper, we have used SFV as a biological pH probe in intact cells, in conjunction with cell fractionation by FFE, to define the acidification of early and late endosomes in CHO cells. The spike glycoproteins of SFV undergo an irreversible conformational change at defined pH values to mediate membrane fusion of the viral envelope with the endosomal membrane (Kielian and Helenius, 1985). By monitoring the low pH-dependent membrane fusion activity of SFV after uptake by baby hamster kidney (BHK-21) cells, it has been possible to show that wild-type virus reaches endosomes of a pH $\leq$6.2 far more rapidly than an SFV mutant (*fus-1*) reaches its fusion pH of $\leq$5.3 (Kielian et al., 1986). In CHO cells, SFV provides an excellent pH-sensitive endocytic tracer because, as indicated above, its transit through kinetically defined and physically distinct endosome subpopulations has been well characterized (Schmid et al., 1988). Since the pH of individual endosomes reached by internalized SFV is scored by an irreversible alteration in the trypsin sensitivity of the E1 spike glycoprotein, the kinetics of endosome acidification in intact CHO cells can be easily quantified and correlated by cell fractionation with the appearance of SFV in physically distinct early and late endosome subpopulations.

We have found that SFV is first internalized into an endocytic compartment of pH >6.2 before delivery to early endosomes which have an average internal pH of $\leq$6.2 but which vary in pH from >6.2 to $\leq$5.3. SFV is then delivered to late endosomes which have an average pH $\leq$5.3. In addition, we compared the kinetics of acidification in wild-type cells with two CHO mutants, B3853 and M311, that represent different genetic complementation groups (*end-1* and *end-2*, respectively) with temperature-sensitive defects in endosomal acidification in vitro (Roff et al., 1986). Finally, since the acidification defect was found to be partially penetrant even at the permissive temperature, we were able to further define the nature of the defect in isolated endosomes.

Materials and Methods

Viruses and Cells

Virus was propagated, plaque purified, stored, and radiolabeled as previously described (Kielian et al., 1984, 1986) using BHK-21 cells. Wild-type CHO cells were maintained in suspension in α-MEM supplemented with 5% FCS (J/R Scientific, Woodland, CA), penicillin, and streptomycin. B3853 (*end-1*) and M311 (*end-2*) mutant CHO cell lines (generously provided by April Robbins, NIH) were grown at 34°C in α-MEM supplemented with 10% FCS, penicillin, and streptomycin.

For experiments, 35-mm dishes were seeded with 1×10^5 wild-type, 2×10^5 B3853, or 2.5×10^5 M311 CHO cells and grown for 2 d at 34°C to ~90% confluency. To initiate a temperature shift, cells were grown for 4 h in a CO_2 (5%) incubator equilibrated to 41°C. Cells temperature shifted by incubating overnight at 39°C showed a less pronounced phenotype (Roff et al., 1986).

Assays for Endocytosis, Degradation, and Intracellar Conversion of Viral E1 Protein

The kinetics of [^{35}S]methionine-labeled virus uptake into CHO cells, degradation in lysosomes to TCA-soluble counts (Marsh and Helenius, 1980; Schmid et al., 1988), and intracellular conversion of the viral E1 glycoprotein to trypsin resistance (Helenius et al., 1985; Kielian et al., 1986) have been previously described. For all assays, radiolabeled virus was prebound to cells on 35-mm plates at 4°C with continuous shaking in RPMI binding media containing 0.2% BSA and buffered with 10 mM Hepes at either pH 6.8 for *wt* SFV or pH 6.5 for *fus-1*. Free virus was removed by washing cells twice with binding media. The cells were rapidly warmed to 37°C by adding pre-warmed media and floating the dishes in a 37°C water bath. At the indicated times, the medium was removed (to measure TCA-soluble radioactivity) and the cells were rapidly cooled by adding ice-cold PBS and by placing the dishes on ice. Surface-bound virus was removed by proteolysis with subtilisin (Sigma Chemical Co., St. Louis, MO; 1 mg/ml in 10 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 8) for 1 h on ice. The cells were collected and washed in 10 ml of PBS by centrifugation and the cell pellets were lysed in 450 μl 1% Triton X-100 in PBS containing 5 μg/ml of unlabeled carrier SFV. After pelleting the nuclei, the lysate was divided into one 50 μl aliquot, for direct counting to determine cell-associated virus, and two 200 μl aliquots, for determining the extent of E1 conversion. To one of these 200 μl aliquots trypsin was added at either 200 μg/ml (for *wt* SFV) or 800 μg/ml (for *fus-1* SFV) in PBS containing 1% Triton X-100. The digests were incubated at 37°C for 20 min (for *wt* SFV) or for 60 min (for *fus-1*). Both 200-μl aliquots were then precipitated with 10% TCA and analyzed by SDS-PAGE on 10% Laemmli gels under nonreducing conditions. Gels were prepared for fluorography using sodium salicylate (Chamberlain, 1979). The gels were quantitated by scanning densitometry of the exposed autoradiogram using a transmission scanning densitometer (model GS300; Hoefer Scientific Instruments, San Francisco, CA) connected to an IBM-XT^R with the integrating software (Model GS350; Hoefer Scientific Instruments). The percent E1 conversion was determined as the ratio of the intensity of the E1 band found in the trypsin-treated aliquot to that in the untreated aliquot. Since surface-bound virus had been removed by subtilisin treatment of intact cells before detergent lysis, these values represented the fraction of intracellular E1 in the acid conformation at each time point.

To monitor the efficiency of the removal of surface-bound virus in each experiment, two sets of cells were maintained at 4°C for 0–2 h after virus binding and harvested with or without subtilisin treatment. Analysis of cell lysates as described above demonstrated that none of the surface-bound virus was converted to the acid form and that >95% of it was removed by subtilisin, as found earlier (Schmid et al., 1988). These values were not subtracted from individual data points but taken as the "zero" time point in each curve.

Cell Fractionation by FFE

^{35}S-SFV (~10^7 cpm in 2.5 ml binding medium) was prebound to a confluent 100-mm plate of CHO cells at 4°C. Free virus was removed and the cells were warmed to 37°C by the addition of prewarmed binding medium and incubated for 2.5 min before the addition of ice-cold PBS and rapid cooling on ice. Surface virus was removed by subtilisin digestion on ice as described above. The SFV-containing cells were mixed with 5×10^7 unlabeled "carrier" CHO cells before homogenization. A microsomal fraction was prepared and fractionated by FFE as described previously (Marsh et al., 1987; Schmid et al., 1988).

Cell-free Acidification Assays

To label endosomes, CHO cells were incubated with the pH-sensitive probe FITC-labeled transferrin (Tfn) as previously described (Roff et al., 1986; Fuchs et al., 1989*a*). A postnuclear supernatant was prepared from cell homogenates and subjected to further fractionation by sucrose gradient centrifugation to obtain an enriched endosomal "float-up" fraction also as previously described (Fuchs et al., 1989*a*). FITC-Tfn-containing endosomes (10–50 μg protein/assay) were diluted into 20 mM Hepes-trimethylammo-

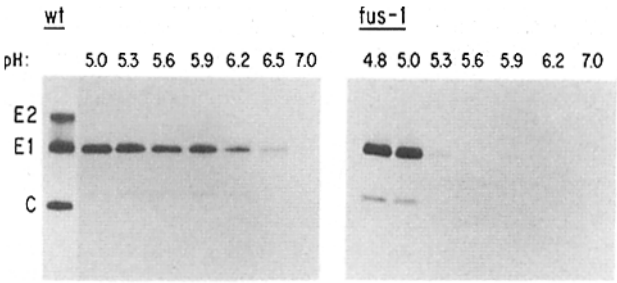

Figure 1. pH dependence of E1 conversion in *wt* and *fus-1* SFV. Aliquots of intact *wt* or *fus-1* virus were incubated for 10 min at 37°C in 20 mM 2,(*N*-morpholino)ethane sulfonic acid, 130 mM NaCl at the indicated pH. The buffer was neutralized by addition of 0.5 M NaOH and digested at 37°C with either 0.1 mg/ml L-1-*p*-tosylamino-2-phenylethyl chlormethyl ketone-trypsin for 20 min (*wt* SFV) or 0.4 mg/ml TPCK-trypsin for 60 min (*fus-1* SFV) in the presence of 1% Triton X-100 as previously described (Kielian and Helenius, 1985). Reactions were terminated by addition of soybean trypsin inhibitor (Sigma Chemical Co.; 2 mg/ml trypsin) and samples processed for gel electrophoresis and fluorography. The control lane on the left shows virus incubated with premixed trypsin and soybean trypsin inhibitor. *E1* and *E2*, the viral spike glycoproteins; *C*, the capsid protein.

nium buffer containing 5 mM $MgSO_4$ and 150 mM of the indicated salts and equilibrated for 2–4 h at 4°C (conditions which completely dissipated any preexisting pH gradients as indicated by fluorescence measurements). The rate of acidification was measured at the ambient temperature by following the decrease in fluorescence after addition of ATP (2.5 mM from a 500-mM stock of Na_2-ATP) using a spectrofluorometer (model LS-5; Perkin-Elmer Corp. Instruments Div., Norwalk, CT) (excitation, 495 nm; emission, 515 nm).

Results

pH Dependence of E1 Conversion in wt and fus-1 SFV

To measure the arrival of endocytosed SFV particles into acidic endosomal compartments, we made use of a pH-dependent conformational change in the spike glycoprotein of SFV. At pH ≤6.2, the E1 subunit of the spike glycoprotein undergoes an irreversible conformational change which renders it resistant to digestion by trypsin (Kielian and Helenius, 1985). By measuring the amount of trypsin-resistant E1 in cell lysates, the exposure of internalized SFV to pH 6.2 can be assayed. Fig. 1 shows the pH titration for the acquisition of trypsin resistance of E1 from *wt* SFV and *fus-1* SFV, an SFV mutant selected for exhibiting a lower pH threshold for membrane fusion (Kielian et al., 1984). *wt* or *fus-1* [^{35}S]-methionine-labeled SFV was incubated for 10 min at 37°C in buffers of various pH, neutralized, and solubilized in Triton X-100 for digestion with trypsin. As seen in Fig. 1, *wt* E1 became resistant to digestion at pH values of ≤6.2 while *fus-1* E1 did not become trypsin resistant until pH ≤5.3. The pH dependence of E1 conversion correlated well with the previously established pH thresholds for the membrane fusion activities of *wt* and *fus-1* SFV (Kielian et al., 1984). The two viruses could therefore be used as biological "pH indicators" to measure the pH of endosomal compartments encountered after internalization of SFV (see also Kielian et al., 1986).

The Kinetics of Arrival of Internalized wt SFV into Acidic Endosomal Compartments

Our previous studies used cell fractionation by FFE and Percoll density gradient centrifugation to establish the kinetics of transit of ^{35}S-SFV between physically distinct early and late endosome subpopulations and of delivery to lysosomes in CHO cells (Schmid et al., 1988). Internalization of the virus and delivery to early endosomes occurred with $t_{1/2}$ = 3–5 min, delivery to late endosomes occurred after a 1–2-min lag with $t_{1/2}$ = 7–10 min, and delivery to lysosomes occurred after a 10-min lag with $t_{1/2}$ = ~35 min (Schmid et al., 1988).

The kinetics of arrival of SFV in an endosomal compartment of pH ≤6.2 were first determined by assessing the trypsin sensitivity of E1 after internalization of *wt* SFV into wild-type CHO cells (Fig. 2 *A*). ^{35}S–SFV was bound to cells in the cold, unbound virus washed away, and the cultures were warmed to 37°C. After the indicated times, remaining cell surface–bound SFV was removed by subtilisin treatment on ice. The cells were then harvested and solubilized in Triton X-100. The lysate was divided into two equal samples one of which was digested with trypsin before both samples were precipitated with TCA and analyzed by SDS-PAGE and autoradiography.

The fraction of trypsin-resistant E1, determined as the ratio of E1 in the trypsin-digested lysate to that in the untreated lysate, indicated that intracellular virus encountered endosomes of pH ≤6.2 with half-maximal conversion at 5 min. These kinetics were consistent with the virus having encountered an average pH ≤6.2 in early endosomes. Surprisingly, even though there was no detectable lag before the first delivery of SFV to an endocytic compartment of pH ≤6.2, at the earliest time points a large percentage of intracellular SFV (70% at 2.5 min) was found in a previously undetected endocytic compartment of pH >6.2. Since the conversion of E1 to the acid conformation in vitro occurs within 10 s (data not shown; Kielian et al., 1986), these kinetics suggest that internalized SFV resides in this compartment for an average of ~5 min before encountering early endosomes of pH ≤6.2. It is unlikely that any conversion of E1 to the acid conformation occurred during the 1-h subtilisin incubation on ice to remove surface-bound virus. In control experiments, identical kinetics were obtained for appearance of the absolute amount of trypsin-resistant E1 if the cells were lysed and trypsin-digested without prior subtilisin treatment (not shown).

SFV Passes through Distinct "Neutral pH" Endocytic Vesicles before Reaching pH ≤6.2 in Early Endosomes and pH ≤5.3 in Late Endosomes

The kinetics for conversion of intracellular E1 to the acid form are a measure of the rate of acidification of individual endosomes, the rate of transfer of SFV to distinct endocytic compartments of lower pH, or a combination of both (Kielian et al., 1986). To distinguish between these possibilities, cell homogenates were analyzed by FFE to resolve endosomal subpopulations and the trypsin sensitivity of E1 in the various fractions was determined. Prebound virus was internalized into early endosomes by incubating CHO cells for 2.5 min at 37°C (Schmid et al., 1988). Remaining surface-bound virus was removed by proteolysis on ice before

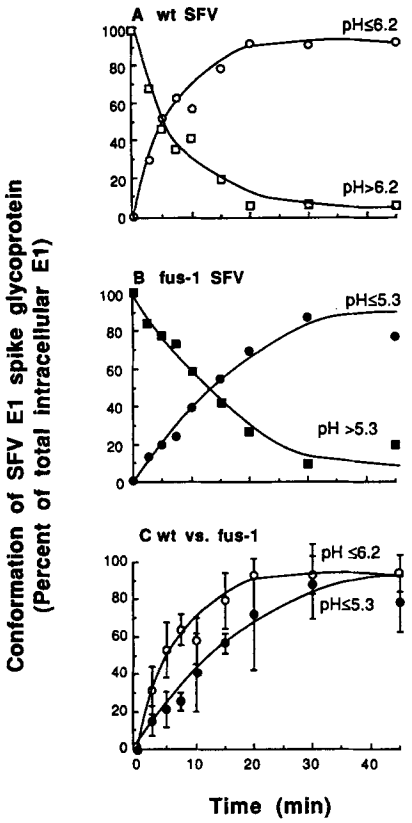

Figure 2. Kinetics of *wt* and *fus-1* E1 in CHO cells. Either *wt* or *fus-1* SFV were bound to CHO cells at 4°C. Unbound virus was removed by washing and the cells were warmed rapidly to 37°C for the indicated times to allow SFV internalization. The plates were then rapidly chilled to 4°C and any remaining surface-bound SFV removed by proteolysis. Cell lysates in 1% Triton X-100 were prepared and the extent of conversion of E1 to the acid form was assessed as described in Fig. 1. (*A*) Time course for the appearance of *wt* SFV in an endocytic compartment of pH ≤6.2. (□) Trypsin-sensitive intracellular *wt* E1; (○) trypsin-resistant intracellular *wt* E1. (*B*) Time course for the appearance of *fus-1* SFV in an endocytic compartment of pH ≤5.3. (■) Trypsin-sensitive intracellular *fus-1* E1; (●) trypsin-resistant intracellular *fus-1* E1. (*C*) A comparison of the kinetics with which intracellular SFV encounters endosomal compartments of pH ≤6.2 and pH ≤5.3. (○) Kinetics of *wt* E1 conversion; (●) kinetics of *fus-1* E1 conversion. Data shown are the average ± SD of four experiments.

the cells were homogenized and a microsomal fraction prepared. When the microsomal pellet was assayed, it was found that 24% of the total internalized E1 was trypsin resistant (not shown, but see Fig. 2 *A*). After FFE, ^{35}S-SFV-containing fractions were identified and collected by centrifugation. The pellets were solubilized in Triton X-100 and assayed for the percentage of E1 converted to the trypsin-resistant acid form.

As shown in Fig. 3, endocytic compartments containing the neutral form of E1 were resolved from early endosomal fractions containing the trypsin-resistant acid form of E1. These results indicate the existence of a previously undetected endocytic compartment of pH >6.2. Unlike early and late endosomes (see Schmid et al., 1988), however, these vesicles were not well resolved from the bulk of the microsomal protein by FFE (not shown). In the more ano-

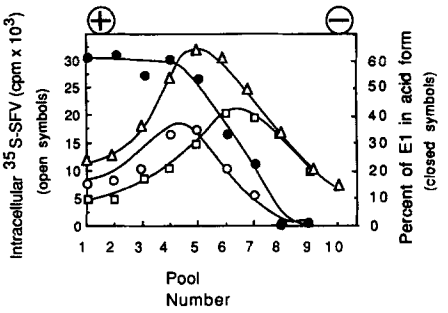

Figure 3. Localization of the site of E1 conversion of internalized SFV by FFE. Prebound ^{35}S-SFV was internalized into CHO cells for 2.5 min at 37°C before the cells were rapidly chilled on ice and any remaining surface-associated virus was removed by proteolysis. The cells were homogenized and a microsomal fraction was prepared for FFE as described in Materials and Methods. After fractionation by FFE, pools of two adjacent ^{35}S-SFV-containing fractions were collected and the membranes pelleted by centrifugation in a rotor (model SW-41; Beckman Instruments, Inc., Fullerton, CA) at 100,000 *g* for 1 h. Membrane pellets were resuspended in 450 μl 1% Triton X-100 in PBS containing 5 μg/ml unlabeled carrier SFV. 50-μl aliquots were counted, and 200-μl aliquots were either digested with trypsin as described in Fig. 1 or untreated. The two samples were then processed for SDS-PAGE and analyzed as described in Materials and Methods. (△) Distribution of total intracellular [^{35}S]methionine-labeled SFV; (□) distribution of trypsin-sensitive E1 (indicating endocytic compartments with pH >6.2); (○) distribution of trypsin-resistant E1 (indicating endocytic compartments with pH ≤6.2); (●) percent tryspin-resistant E1 in each pool.

dally shifted early endosome fractions, 60% of the internalized E1 had converted to the acid form. Thus, although most early endosomes containing virus are capable of acidification to an internal pH ≤6.2, internalized SFV appears first to be localized in endocytic vesicles with an average internal pH >6.2.

The arrival of virus at an endocytic compartment of pH ≤5.3 was next measured by determining the trypsin sensitivity of the E1 glycoprotein after internalization of the mutant virus, ^{35}S-*fus-1*. Fig. 2 *C* shows a comparison of the kinetics of conversion of *wt* and *fus-1* E1 after internalization into wild-type CHO cells. As found previously in BHK-21 cells using a virus penetration assay (Kielian et al., 1986), the kinetics of arrival in an endosomal compartment of pH ≤5.3 were significantly slower ($t_{1/2}$ = ~11 min) than arrival in the less acidic early endosomes. These results indicate that SFV encountered gradually increasing acidity as it traversed the endocytic pathway. Since the half-time for conversion of *fus-1* E1 to the acid conformation in vitro is ~3 min (data not shown; Kielian et al., 1986), these data indicated that internalized SFV arrived in an endosomal compartment of pH ≤5.3 with $t_{1/2}$ = ~8 min. When compared to the kinetics of delivery of internalized SFV into late endosomes (see above; Schmid et al., 1988), these data are consistent with the bulk of *fus-1* E1 conversion occurring in the late endosome subpopulation. As described above for *wt* SFV, identical kinetics for the absolute appearance of trypsin-resistant *fus-1* E1 were obtained if surface-bound virus was not removed; hence, conversion to the acid conformation did not occur during the 1-h (0°C) subtilisin digestion used to remove virus remaining on the plasma membrane.

Table I. Kinetics of SFV Internalization and Transport along the Endocytic Pathway in Mutant and Wild-Type CHO Cells

Cell type	Internalization* ($t_{1/2}$, min)		Arrival in late endosomes‡ (% after 4 min)		Arrival in lysosomes§ (lag, min)	
	34°C	41°C	34°C	41°C	34°C	41°C
Wild type	5′	5′	49%	ND	11′	11′
B3853 (*end-1*)	6′	6′	50%	43%	13′	13′
M311 (*end-2*)	5′	5′	ND	ND	12′	11′

* Determined as cell associated ^{35}S-SFV after subtilisin digestion of cells at 4°C. Data are the average of four experiments.
‡ Determined as the fraction of intracellular ^{35}S-SFV in the more anodally shifted "late" endosomal fractions after FFE.
§ Determined as the first appearance of TCA-soluble [^{35}S]methionine in the media. Data are the average of four experiments.

Even though after 5 min a large fraction of intracellular SFV (~80%) was still present in an endocytic compartment of pH >5.3, the absence of a lag period before conversion of *fus-1* E1 to the acid form (Fig. 2 *B*) suggested either that some SFV is delivered directly to more acidic late endosomes after internalization or that a small population of early endosomes is capable of acidification to pH ≤5.3. To distinguish between these possibilities and to further examine the mechanism of pH regulation of endosomes in intact cells, we next used *wt* and *fus-1* SFV to probe the acidification properties of endosomes in mutant CHO cells with temperature-sensitive defects in endosomal acidification.

Both Early and Late Endosome Acidification Is Defective in Mutant CHO Cells

Endosomal acidification in the B3853 and M311 temperature-sensitive acidification mutants (*end-1* and *end-2* complementation groups, respectively) was examined by following the conversion of E1 to the trypsin-resistant acid conformation after internalization of *wt* or *fus-1* SFV. Although the kinetics of internalization, transit through early and late endosomes and delivery to lysosomes for both viruses were indistinguishable in wild-type and mutant cell lines (Table I), the rates for both *wt* and *fus-1* E1 conversion were substantially reduced in both mutants. These data are presented in Fig. 4 and summarized in Table II.

While the extent to which *wt* E1 acquired the trypsin-resistant acid conformation was similar in all three cell lines (Fig. 4 *A*), the kinetics of conversion at the permissive temperature were slightly delayed in both the B3853 and M311 cells ($t_{1/2}$ = ~8 min compared to $t_{1/2}$ = ~5 min in wild-type cells). The slight defect in endosome acidification to pH ≤6.2 in intact cells detected at the permissive temperature was even more apparent when the conversion of *fus-1* E1 was measured. As shown in Fig. 4 *B*, both the rate and extent of

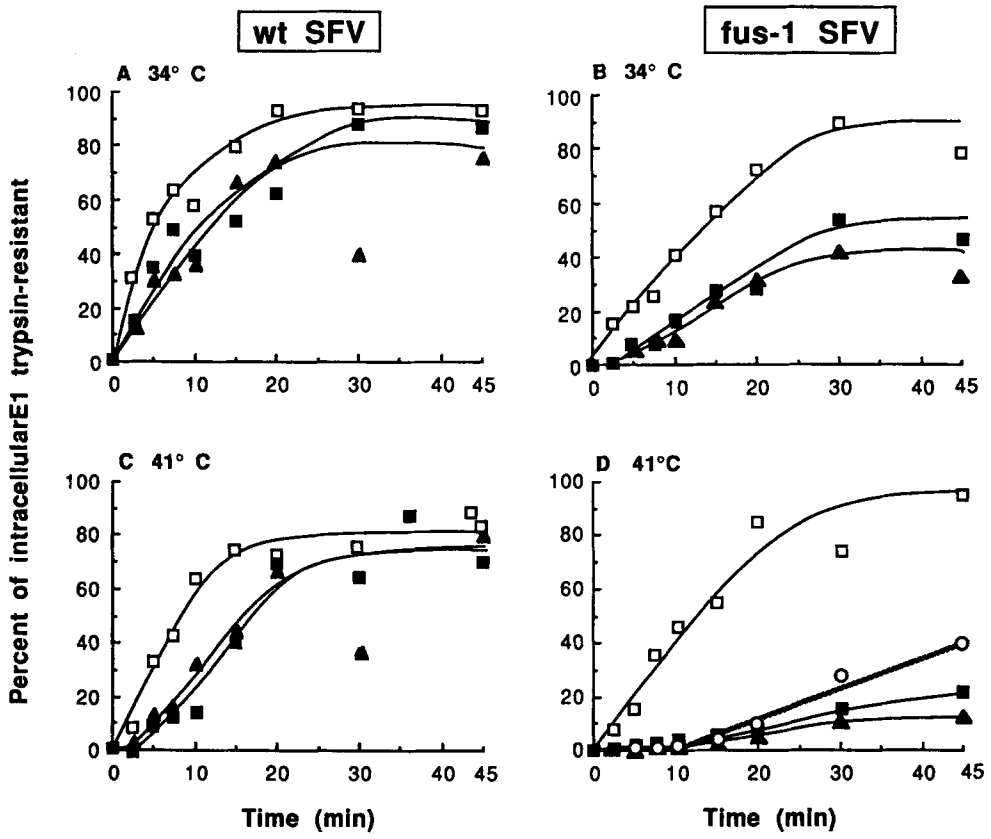

Figure 4. Kinetics of *wt* and *fus-1* E1 conversion in mutant and wild-type CHO cells. The extent of E1 conversion of intracellular *wt* and *fus-1* SFV into wild-type CHO cells (□), B3853 (■), and M311 (▲) was determined as described in Fig. 2. Cells were maintained at the permissive temperature (34°C) before internalization of *wt* (*A*) or *fus-1* (*C*) SFV. Virus was prebound to cells at 4°C before incubation at 37°C for the indicated times before the extent of E1 conversion was assessed. Cells were shifted to the restrictive temperature (41°C) for 4 h before binding virus at 4°C and internalization of *wt* (*B*) or *fus-1* (*D*) SFV at 39°C for the indicated times. In *D* for comparison, the stippled line (○) shows the kinetics of delivery to lysosomes in either B3853 or M311 cells as assessed by the appearance of TCA-soluble [^{35}S]methionine in the media. In control experiments the kinetics for SFV internalization and degradation at either 37 or 39°C were indistinguishable. Data shown are the average of three experiments.

Table II. Kinetics of SFV E1 Conversion in Wild-Type and Mutant CHO Cells

Cell type	*wt* SFV (pH ≤6.2) $t_{1/2}$ [lag]		*fus-1* SFV (pH ≤5.4) $t_{1/2}$ [lag]	
	34°C	41°C	34°C	41°C
Wild type	5 min [0]	5.5 min [0]	11 min [0]	11.5 min [0]
B3853 (*end-1*)	8 min [0]	13 min [~2']	15 min [2–3']	23 min [10']
M311 (*end-2*)	8 min [0]	14.5 min [~2']	16 min [2–3']	25 min [10']

fus-1 E1 conversion were markedly reduced in B3853 and M311 mutants as compared to wild-type CHO cells. After internalization, there was a lag period of ~2 min before the first appearance of trypsin-resistant *fus-1* E1 in both mutant cell lines. The appearance of a lag suggested that SFV must pass through an early endosomal subpopulation incapable of attaining a pH ≤5.3 before delivery to late endosomes with pH ≤5.3. In addition, the extent of *fus-1* E1 conversion was reduced, suggesting that almost 50% of the *fus-1* SFV was delivered to lysosomes and degraded without ever having encountered a sufficiently low pH environment to mediate E1 conversion. Together, these data indicate that the temperature-sensitive acidification defect in B3853 and M311 mutants is partially penetrant at the permissive temperature.

To assess the extent of the temperature-sensitive defect in endosomal acidification in B3853 and M311 CHO cell mutants, the kinetics of conversion *wt* and *fus-1* E1 were next measured in cells grown under nonpermissive conditions. Although preincubating wild-type cells for 4 h at 41°C before binding and internalization of SFV had no effect on the rate of E1 conversion (assayed at 37°C), the kinetics in both B3853 and M311 mutants were markedly reduced (Fig. 4, *C* and *D*). Trypsin-resistant *wt* E1 appeared only after a lag of 1–2 min, suggesting that early endosomes in mutant cells at the nonpermissive temperature could not generate an internal pH ≤6.2. The half-maximal conversion of *wt* E1 occurred only after 13–15 min in the B3853 and M311 mutants compared to 5.5 min in wild-type cells at 41°C. Since the rate of transit of SFV along the endocytic pathway was unchanged (Table I), and since the extent of *wt* E1 conversion was not reduced in either mutant line at 41°C (Fig. 4 *C*), these kinetics suggested that at the nonpermissive temperature ^{35}S-SFV encountered a compartment of pH ≤6.2 only after arrival in late endosomes in the mutant cells.

In contrast, at the nonpermissive temperature, both the rate and extent of *fus-1* E1 conversion were severely reduced (Fig. 4 *D*). After a prolonged lag of >10 min, only 15% of internalized *fus-1* E1 became trypsin resistant. These results suggest that at the nonpermissive temperature the bulk of *fus-1* SFV was degraded in lysosomes without having reached an endocytic compartment of pH ≤5.3. The kinetics of delivery of *fus-1* SFV to lysosomes in B3853 and M311 cells, as indicated by the appearance of TCA-soluble [^{35}S]methionine in the media, are given in Fig. 4 *D* (*stippled line*) for comparison.

Acidification of Endosomes Isolated from Mutant and Wild-Type CHO Cells

Our data have thus far demonstrated that at the nonpermissive temperature, acidification of both early and late endosomes in intact cells is severely defective in the temperature-sensitive mutants B3853 and M311, and that this defect is partially penetrant at the permissive temperature. Previous results had shown that this defect in endosomal acidification can also be detected in cell-free acidification assays using endosomes isolated from mutant cells grown at the nonpermissive temperature (Roff et al., 1986). However, the actual mechanism of the acidification defect has remained poorly defined. To further characterize the nature of the acidification defect, we next evaluated the acidification properties and ion permeabilities of endosomes isolated from mutant cells maintained at the permissive and nonpermissive temperatures.

Wild-type and mutant CHO cells were maintained at 34 or 41°C for 4 h and labeled with FITC-Tfn (a selective marker for early endosomes [Schmid et al., 1988]) before homogenization and isolation of an enriched endosome fraction by sucrose density gradient centrifugation (Roff et al., 1986; Fuchs et al., 1989*a*). After equilibrating the labeled endosomes in KCl-containing buffer to dissipate any preexisting ion gradients (see Materials and Methods), ATP-dependent acidification was determined by measuring the quenching of FITC fluorescence (Galloway et al., 1983; Roff et al., 1986; Schmid et al., 1988). As shown previously (Roff et al., 1986), acidification of Tfn-labeled endosomes from temperature-sensitive B3853 and M311 mutants was completely inhibited after growth at 41°C. The acidification of endosomes from wild-type CHO cells was not affected.

Since the endosomal proton pump in CHO cells is electrogenic (i.e., it generates an interior-positive membrane potential which opposes continued proton translocation), influx of permeant external anions (e.g., Cl^-) or efflux of internal alkali cations (e.g., K^+) is required for acidification (Fuchs et al., 1989*a*,*b*). To determine whether the defective acidification seen in endosomes from mutant cells grown at 41°C might be due to defective anion or cation permeabilities, acidification assays were performed in K^+-containing medium in the presence of the K^+ ionophore valinomycin, which would be expected to shunt any membrane potential built up by the proton ATPase in the presence of an impermeant anion (Fig. 5). The inability of valinomycin to reverse the acidification defect demonstrates that the mutant phenotype does not reflect impaired permeabilities for either K^+ or Cl^-.

Acidification of Endosomes Isolated from Cells Grown at the Permissive Temperature

The low level of acidification activity observed in vitro for endosomes isolated from mutant cells grown at the nonpermissive temperature made it impossible to further study the nature of this defect. However, the fact that intact cells seemed to exhibit a partially affected acidification phenotype even at the permissive temperature (Table II) suggested that

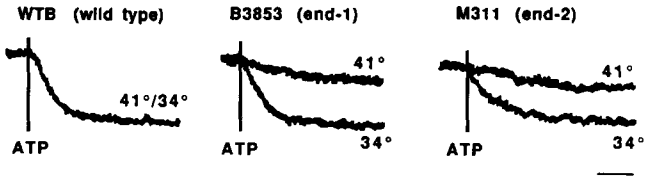

Figure 5. The defect in in vitro endosome acidification in B3853 and M311 cells is not due to changes in ion conductances. Endosomes, labeled with FITC-Tfn, were isolated by sucrose gradient centrifugation from wild-type, B3853, and M311 CHO cells grown at 34 or 41°C as described in Materials and Methods. Isolated endosomes were equilibrated in a buffer containing 150 mM KCl, 20 mM Hepes trimethylammonium, pH 7.4, and 1 μM valinomycin to facilitate K^+ conductance down a membrane potential gradient. ATP-dependent endosomal acidification was monitored by the fluorescent quenching of FITC as described in Materials and Methods. Acidification of endosomes from wild-type cells was unaffected at 41°C (*left trace*). The defect in acidification of endosomes isolated from mutant cells grown at 41°C was not rescued by the presence of valinomycin (*right and middle traces*). Bar, 5 min.

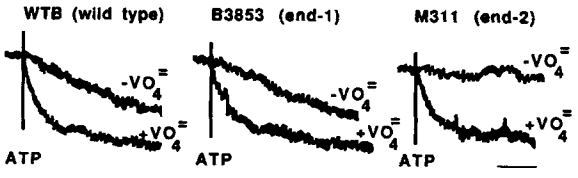

Figure 7. Sensitivity of acidification to Na/K-ATPase activity in endosomes from acidification-defective mutant cells. FITC-Tfn-labeled endosomes were prepared from wild-type, B3853, and M311 CHO cells grown at 34°C as in Fig. 5. Isolated endosomes were equilibrated in Na/K buffer (125 mM NaCl, 25 mM KCl) with or without 1 mM Na_2VO_4. 2.5 mM ATP was then added and acidification monitored as described in Materials and Methods. Endosomes from wild-type cells and the mutant B3853 displayed the same partial sensitivity to inhibition by Na/K buffer and the complete reversal of this inhibition by VO_4 (*left and middle traces*). In contrast, ATP-dependent acidification of endosomes from M311 cells was completely blocked in Na/K buffer, although complete reversal of this inhibition was still obtained by addition of Na_2VO_4 (*right trace*). Bar, 5 min.

endosomes isolated from mutant cells grown at 34°C might similarly exhibit a partial defect and could therefore be used to further characterize the basis for the mutant phenotype.

To determine whether defective acidification in the mutant CHO cells was due to increased H^+ permeability, we first compared H^+ flux in endosomes isolated from wild-type and mutant cells grown at 34°C. As shown in Fig. 6, when ATP was added to FITC-Tfn-containing endosomes, roughly equivalent amounts of acidification were observed for wild-type endosomes (Fig. 6 *A*) as well as for endosomes from B3858 cells (*end-1*; Fig. 6 *B*), and M311 cells (*end-2*; Fig. 6 *C*). After maximum acidification was reached, ATP was "removed" by the addition of hexokinase-glucose and the rates of dissipation of each pH gradient were monitored. Since the rate of H^+ efflux from mutant endosomes was no more rapid than that observed for wild-type endosomes, it was unlikely that the reduced acidification capacity in B3853 or M311 cells reflected an increased H^+ permeability at either permissive or nonpermissive temperatures.

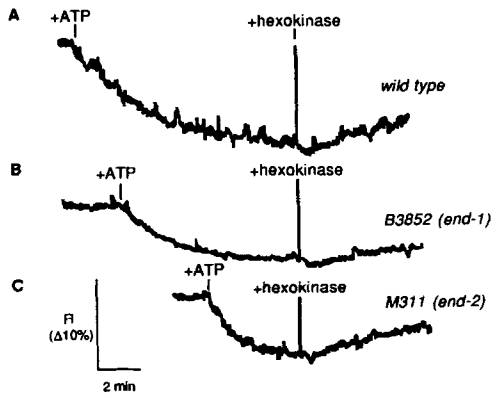

Figure 6. The defect in in vitro endosome acidification in B3853 and M311 cells is not due to changes in H^+ permeability. FITC-Tfn-labeled endosomes were isolated (as in Fig. 5) from wild-type, B3853, and M311 CHO cells maintained at 34°C. Labeled endosomes were then equilibrated (2–4 h at 0°C) in a buffer containing 150 mM KCl, 20 mM Hepes-trimethylammonium, pH 7.4. After transfer to spectrofluorometer cuvette, ATP was added (2.5 mM) to permit acidification. After maximum acidification had been achieved (5–10 min), ATP was "removed" by the addition of yeast hexokinase (Sigma Chemical Co.; 12 U/ml) and 5 mM glucose, conditions which would effectively deplete ATP by hydrolysis. The rate of dissipation of the pH gradient due to H^+ efflux was monitored as a progressive increase in FITC fluorescence as a function of time. Neither mutant cell line exhibited an increased rate of H^+ efflux relative to controls. Each trace represents the acidification signal obtained from ~50 μg of protein in an endosome-enriched gradient fraction, containing similar amounts of FITC-Tfn. Vertical bar, 10% change in the initial fluorescence; horizontal bar, 2 min.

We next determined whether the acidification of endosomes isolated from B3853 and M311 mutants (grown at 34°C) might exhibit an increased sensitivity to conditions expected to affect the endosomal membrane potential. We found previously that endosomes from wild-type CHO cells contain a functional Na^+,K^+-ATPase activity which could oppose ATP-dependent acidification by contributing to an interior-positive membrane potential (possibly due to electrogenic Na^+ transport) (Fuchs et al., 1989*a*). Thus, when equilibrated in media containing both Na^+ and K^+, acidification of endosomes from wild-type cells is partially decreased; the addition of Na_2VO_4 or ouabain (selective inhibitors of the NA^+,K^+-ATPase) reverses the inhibition of acidification (Fuchs et al., 1989*a*).

As shown in Fig. 7, ATP-dependent acidification of endosomes from the *end-2* cell line, M311, was found to be hypersensitive to conditions which favor NA^+,K^+-ATPase activity. Acidification was completely inhibited in buffer containing Na^+ and K^+ (Fig. 7, *right*) relative to acidification activity for M311 endosomes in K^+ alone (Fig. 5, *right*). This inhibition was completely reversed by the addition of Na_2VO_4 (Fig. 7).

That the pronounced inhibition of M311 endosome acidification in Na^+ and K^+-containing buffer was due to a differential sensitivity to membrane potential, and not due to an altered Na^+,K^+-ATPase, was supported by the fact that M311 endosome acidification also exhibited an increased dependence on external permeant anions. This was demonstrated

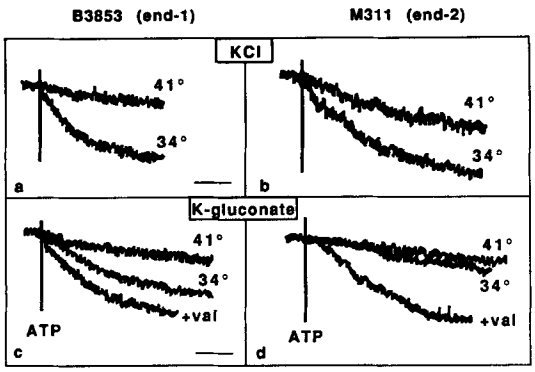

Figure 8. Anion dependence of ATP-dependent endosome acidification in temperature-sensitive mutant cells. FITC–Tfn-containing endosomes were prepared from B3853 (*end1*) and M311 (*end2*) cells grown and labeled at either permissive (34°C) or nonpermissive (41°C) temperatures. Endosomes were equilibrated in either KCl buffer or K^+-gluconate buffer in the presence or absence of 1 μM valinomycin (*val*). ATP was added at the indicated times to initiate acidification. KCl buffer: acidification of endosomes taken from the mutant B3853 (*A*) or M311 (*B*) grown at 41 or 34°C. K^+-gluconate buffer: 41 and 34°C endosomes from B3853 cells (*C*) or M311 cells (*D*), and 34°C endosomes assayed in the presence of valinomycin. Bar, 5 min.

by replacing Cl^- with the relatively impermeant organic anion gluconate. In contrast to the partial inhibition exhibited by endosomes isolated from wild-type CHO cells (Fuchs et al., 1989*a*) or from the mutant B3853 (grown at 34°C) (Fig. 8, *a* and *c*), replacement of KCl with K^+-gluconate almost completely blocked the ATP-dependent acidification of M311 endosomes (Fig. 8, *b* and *d*). The extent of this inhibition rendered endosomes from M311 cells grown at the permissive temperature as acidification incompetent as endosomes from M311 cells grown at the nonpermissive temperature (Fig. 8 *d*).

Our data indicates that the genetic lesion in M311 cells is not due to alterations in the ion permeabilities of the endosomal membrane but most likely results from a direct or indirect effect on the endosomal H^+ pump itself. This defect appears to render the H^+-ATPase in M311 endosomes relatively incapable of transporting protons against an electrical gradient.

Discussion

SFV as a pH-sensitive Endocytic Tracer in CHO Cells

We have used *wt* SFV and the *fus-1* SFV mutant (Kielian et al., 1984, 1986) which undergo irreversible conformational changes at pH ≤6.2 and pH ≤5.3, respectively, as endocytic tracers to measure endosomal pH in intact cells. SFV provides an excellent pH-sensitive endocytic tracer in CHO cells because its transit through kinetically defined and physically distinct endosome subpopulations has been well defined (Schmid et al., 1988). The sensitivity of the E1 trypsin-resistance assay, coupled with the irreversibility of the acid-induced conformational change, permits the recording of the earliest encounter of internalized radiolabeled SFV with endocytic compartments of defined pH. With the development of acid conformation-specific antibodies to SFV spike glycoproteins (Kielian, M., unpublished results) and the use of other viruses such as influenza for which acid conformation-specific antibodies are available (Copeland et al., 1987, 1988), this technique should also enable morphological identification of endosomal compartments of defined pH.

Acidification Kinetics Define the Existence of Early and Late Endosomes in Intact Cells

A number of studies have shown that endocytic tracers encounter increasingly acidic environments as they are transported to lysosomes (Murphy et al., 1984; Kielian et al., 1986; Yamashiro and Maxfield, 1987). In most cases, these studies have used the acid-dependent alterations in FITC fluorescence to determine the pH of endocytic vesicles as a function of time after internalization. Our results confirm measurements—obtained using cell populations by spectrofluorometry (Merion et al., 1983), large numbers of individual cells by flow cytofluorometry (Murphy et al., 1984), or single cells by fluorescence microscopy (Yamashiro and Maxfield, 1987)—which have all provided evidence that internalized tracers rapidly (within 10 min) reach a slightly acidic compartment of pH 6.0–6.5 and then more slowly encounter pH ≤5.0.

We have extended these observations, however, by combining kinetic measurements of pH with detailed information describing kinetics of transport of endocytic tracers between distinct subpopulations of endosomes and from endosomes to lysosomes. In addition, we have demonstrated that a physically distinct endocytic compartment is encountered rapidly after internalization which has a relatively neutral pH. In general, using optical methods alone, it has proved difficult to detect the acidification of this earliest population of endocytic vesicles (Murphy et al., 1984; Yamashiro and Maxfield, 1987). Only Sipe and Murphy (1987) have previously detected a 1–2-min lag between internalization and acidification of FITC-Tfn in their measurements of endosome acidification in intact cells using flow cytofluorometry.

By combining a sensitive and quantitative biochemical assay for acidification with detailed information concerning SFV transport through the endocytic pathway in CHO cells, we have been able to both confirm earlier suggestions and, more importantly, to provide direct evidence for the existence of distinct subpopulations with characteristic internal pHs. Thus, in wild-type CHO cells, we have shown that SFV is first internalized into an endocytic compartment of pH >6.2 before delivery to early endosomes ($t_{1/2}$ = ~5 min) where it encounters an average pH of ≤6.2. These relatively neutral endocytic compartments which contain newly internalized virus were resolved from acidic early endosomes by FFE. Since we have previously shown that anodally shifted early endosomes are compositionally distinct from the plasma membrane (Schmid et al., 1988), this relatively neutral endocytic compartment could represent primary endocytic vesicles which are derived from the plasma membrane. Alternatively, or in addition, during this 5-min period SFV could be present in incompletely sealed coated pits which are inaccessible to extracellular proteases and which, upon homogenization, vesiculate to form structures which are resolved from more acidic early endosomes. Given the relatively long half-time (5 min) before arrival in an endosomal compartment of pH ≤6.2, compared to the estimated lifetime

(<1 min) of a coated pit (Pearse and Bretscher, 1981), it is unlikely that these structures could account for all of the cell-associated neutral SFV. An average pH of ≤5.3 is encountered with kinetics consistent with the kinetics of arrival of SFV in physically distinct late endosomes ($t_{1/2}$ = ~8–10 min) (Schmid et al., 1988).

These results are in agreement with our previous results using a similar approach to follow the kinetics and extent of endosome acidification in BHK-21 cells (Kielian et al., 1986). However, since surface-bound SFV was not removed in these experiments, we were unable to detect the earliest neutral endocytic compartment. In addition, the kinetics of transit through the endocytic pathway in BHK-21 cells are slower and more asynchronous at each stage (uptake, endosome acidification, and delivery to lysosomes) compared with those in CHO cells. A physical distinction between early and late endosomes has not yet been attempted for BHK-21 cells and the reasons for the kinetic differences between these two cell types are unknown.

SFV Must Pass through Early Endosomes before Reaching Late Endosomes

In wild-type CHO cells, *fus-1* SFV was found to encounter endosomes of pH ≤5.3 almost immediately after internalization. As discussed earlier (Kielian et al., 1986), the absence of a detectable lag suggests either that a fraction of early endosomes have an internal pH as low as 5.3 or that a fraction of the incoming SFV "by-passes" early endosomes and is delivered directly to late endosomes. The kinetics of *fus-1* E1 acid conversion after endocytosis into acidification-defective cell lines appears to distinguish between these two possibilities. When measured at the permissive temperature, *fus-1* SFV encountered a compartment of pH ≤5.3 only after an ~2 min lag, suggesting that in neither B3853 nor M311 cells could early endosomes generate a pH of ≤5.3. Conversion to the acid conformation occurred rapidly (i.e., before delivery to lysosomes) and efficiently after delivery to late endosomes, a step shown by FFE to require a similar lag period of 2–3 min (Schmid et al., 1988). Thus, at least in these two cell lines, incoming virus was not by-passing early endosomes to reach the pH 5.3 late endosomes, otherwise E1 conversion to the acid conformation would still have been expected to occur without a lag.

In summary, our data indicate that SFV is first internalized into a relatively neutral (pH >6.2) endocytic compartment before delivery to early endosomes. The average pH of early endosomes is ≤6.2 but they appear to have a broad pH range of >6.2 to ≤5.3. Upon arrival in physically and biochemically distinct late endosomes, internalized SFV encounters an average lumenal pH ≤5.3.

Acidification in Intact Acidification-defective Mutant CHO Cells

Endosome acidification, as indicated by conversion of SFV E1 to the acid conformation, was slowed in the mutant CHO cell lines even at the permissive temperature of 34°C. The partial penetrance has also been observed for other B3853 and M311 phenotypes such as mannose 6-phosphate receptor-mediated endocytosis, sensitivity to toxins, and terminal glycosylation, all of which are slightly reduced in the mutants at the permissive temperature relative to wild-type controls (Roff et al., 1986).

At the nonpermissive temperature, our finding that acidification of both early and late endosomes is defective differs from that reported by Yamashiro and Maxfield (1987) for nonconditional alleles of the same two complementation groups (see Robbins et al., 1984). Using a new microspectrofluorometry method, they found that acidification of endosomes encountered early (i.e., within 3–10 min) after internalization of FITC-dextran was reduced in both mutants; in contrast, acidification of later endosomal compartments (encountered after 10–15 min of uptake) was nearly normal. Acidification in a third class of endosomes, identified morphologically in CHO cells, which are involved in the recycling of Tfn receptors to the cell surface after segregation from ligands en route to lysosomes, was also unaffected (Yamashiro and Maxfield, 1987).

These results could reflect differences in the extent of the temperature-sensitive vs. nonconditional phenotypes (e.g., the existence of compensatory adaptations in endosomal acidification properties enabling survival of the nonconditional mutations of the same two genes). Alternatively, or in addition, the discrepancy could reflect differences in the techniques used. Because of the lower sensitivity achieved by the use of fluoresceinated ligands to study endosomal acidification, microspectrofluorometry did not permit the definition of detailed kinetics for endosomal acidification; instead, average pH values were obtained for endosomes labeled during a 5-min pulse of FITC-dextran and subsequent (1–10 min) chase periods. Moreover, under the labeling conditions used by Yamashiro and Maxfield (1987) (6–15-min uptake periods), cell fractionation by FFE indicated that endocytic tracers are distributed among both early and late endosomes, as well as lysosomes (Schmid et al., 1988). Since lysosomal acidification is unaffected in these mutants (Robbins et al., 1984; Roff et al., 1986), the possible contributions of labeled lysosomes to a positive acidification signal (especially at the later time points) cannot be ruled out. SFV cannot be used to measure lysosomal pH due to rapid degradation of the spike glycoproteins upon delivery to lysosomes.

Properties of Acidification of Endosomes Isolated from Mutant Cells

Our earlier studies have demonstrated that the endosomal proton pump is electrogenic and therefore susceptible to regulation by changes in membrane potential (Mellman et al., 1986; Fuchs et al., 1989*a*,*b*). Endosomal membrane potential could, in turn, be regulated by alterations in ion permeabilities of the endosomal membrane or by changes in activities of other electrogenic pumps present in the endosomal membrane. We have found that neither the B3853 nor M311 defect causes pronounced changes in endosomal membrane permeability to anions, cations, or protons. Thus, it would appear that both of these mutations directly or indirectly affect the activity of proton pump itself.

In the case of at least one cell line of the *end-2* complementation group, M311, we were able to obtain additional evidence supporting a proton pump defect. Endosomes from M311 cells maintained at the permissive temperature exhibited a marked increase in sensitivity to conditions which would be expected to effect endosomal membrane potential (i.e., replacing permeant external anions with relatively impermeant species or equilibrating the endosomes in buffer containing Na^+ and K^+ under conditions which favor the activity of the Na^+,K^+-ATPase). The simplest explanation of

these results is that M311 cells contain an endosomal proton pump which is abnormally sensitive to alterations in membrane potential. Additional information will be necessary to establish the suggested relationship between membrane potential and proton pump activity in wild-type or mutant cells. However, the fact that a mutation resulting in defective endosome acidification in intact cells can be correlated with an apparently increased sensitivity to pH regulation by changes in membrane potential in vitro supports the possibility that membrane potential may play a physiological role in controlling endosomal pH. This is consistent with our previous finding that early endosomes not only exhibit a reduced capacity for ATP-dependent acidification in vitro relative to late endosomes or lysosomes (Schmid et al., 1988), but also uniquely contain a Na^+,K^+-ATPase activity which may further limit their acidification by an electrogenic mechanism (Fuchs et al., 1989*a*).

We wish to thank Dr. April Robbins (National Institutes of Health) for her gift of the CHO cell mutants and for helpful discussions. M. C. Kielian wishes to thank Kathleen Ullrich for her excellent technical assistance.

This work was supported by National Institutes of Health grants to A. Helenius and I. Mellman and by a grant from the Lucille P. Markey Charitable Trust. M. C. Kielian was supported by National Institutes of Health research grant GM38743, Cancer Center Core grant CA13330, and Biomedical Research Support Grant Program funds (SO7RR05397-25). S. L. Schmid is a Lucille P. Markey Scholar.

Received for publication 2 August 1988 and in revised form 2 December 1988.

References

Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with water-soluble fluor, sodium salicylate. *Anal. Biochem.* 98:132–135.

Copeland, C. S., R. W. Doms, E. M. Bolzau, R. G. Webster, and A. Helenius. 1987. Assembly of influenza hemagglutinin trimers and its role in intracellular transport. *J. Cell Biol.* 103:1179–1191.

Copeland, C. S., K.-P. Zimmer, K. R. Wagner, G. A. Healey, I. Mellman, and A. Helenius. 1988. Folding, trimerization, and transport are sequential events in biogenesis of influenza virus hemagglutinin. *Cell.* 53:197–209.

Davis, C. G., J. L. Goldstein, T. C. Sudhof, R. G. W. Anderson, D. W. Russell, and M. S. Brown. 1987. Acid-dependent ligand dissociation and recycling of LDL receptor mediated by the growth factor homology region. *Nature (Lond.).* 326:760–765.

Fuchs, R., S. Schmid, and I. Mellman. 1989*a*. A possible role for Na^+,K^+-ATPase in regulating ATP-dependent endosome acidification. *Proc. Natl. Acad. Sci. USA.* 86:539–543.

Fuchs, R., P. Mâle, and I. Mellman. 1989*b*. Acidification and ion permeabilities of highly purified endosomes isolated from rat liver by free flow electrophoresis. *J. Biol. Chem.* In press.

Galloway, C. J., G. E. Dean, M. Marsh, G. Rudnick, and I. Mellman. 1983. Acidification of macrophage and fibroblast endocytic vesicles in vitro. *Proc. Natl. Acad. Sci. USA.* 80:3334–3338.

Helenius, A., I. Mellman, D. Wall, and A. Hubbard. 1983. Endosomes. *Trends Biochem. Sci.* 8:245–250.

Helenius, A., M. Kielian, J. Wellsteed, I. Mellman, and G. Rudnick. 1985. Effects of monovalent cations on Semliki Forest virus entry into BHK-21 cells. *J. Biol. Chem.* 260:5691–5697.

Kielian, M. C., and A. Helenius. 1985. pH-induced alterations in the fusogenic spike protein of Semliki Forest virus. *J. Cell Biol.* 101:2284–2291.

Kielian, M. C., S. Keränen, L. Kääriäinen, and A. Helenius. 1984. Membrane fusion mutants of Semliki Forest virus. *J. Cell Biol.* 98:139–145.

Kielian, M. C., M. Marsh, and A. Helenius. 1986. Kinetics of endosome acidification detected by mutant and wild-type Semliki Forest virus. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:3103–3109.

Marsh, M., and A. Helenius. 1980. Adsorptive endocytosis of Semliki Forest virus. *J. Mol. Biol.* 142:439–454.

Marsh, M., S. Schmid, H. Kern, E. Harms, P. Mâle, I. Mellman, and A. Helenius. 1987. Rapid analytical and preparative isolation of functional endosomes by free flow electrophoresis. *J. Cell Biol.* 104:875–886.

Mellman, I., R. Fuchs, and A. Helenius. 1986. Acidification of the endocytic and exocytic pathways. *Annu. Rev. Biochem.* 55:712–722.

Merion, M., P. Schlesinger, R. M. Brooks, J. M. Moerhing, T. J. Moerhing, and W. S. Sly. 1983. Defective acidification of endosomes in Chinese hamster ovary cell mutants "cross-resistant" to toxins and viruses. *Proc. Natl. Acad. Sci. USA.* 80:5315–5319.

Murphy, R. F., S. Powers, and C. R. Cantor. 1984. Endosome pH measured in single cells by dual fluorescence flow cytometry: rapid acidification of insulin to pH 6. *J. Cell Biol.* 98:1757–1762.

Pearse, B. M. F., and M. S. Bretscher. 1981. Membrane recycling by coated vesicles. *Annu. Rev. Biochem.* 50:85–101.

Robbins, A. R., C. Oliver, J. L. Bateman, S. S. Krag, C. J. Galloway, and I. Mellman. 1984. A single mutation in Chinese hamster ovary cells impairs both Golgi and endosomal functions. *J. Cell Biol.* 99:1296–1308.

Roff, C. F., R. Fuchs, I. Mellman, and A. R. Robbins. 1986. Chinese hamster ovary cell mutants with temperature-sensitive defects in endocytosis. I. Loss of functions on shifting to the nonpermissive temperature. *J. Cell Biol.* 103:2283–2297.

Schmid, S. L., R. Fuchs, P. Mâle, and I. Mellman. 1988. Two distinct subpopulations of endosomes involved in membrane recycling and transport to lysosomes. *Cell.* 52:73–83.

Sipe, D. M., and R. F. Murphy. 1987. High resolution kinetics of transferrin acidification in Balb/c 3T32 cells: exposure to pH 6 followed by temperature-sensitive alkalinization during recycling. *Proc. Natl. Acad. Sci. USA.* 84:7119–7123.

Yamashiro, D. J., and F. R. Maxfield. 1987. Kinetics of endosome acidification in mutant and wild-type Chinese hamster ovary cells. *J. Cell Biol.* 105: 2713–2721.