

Uncoating of Human Rhinovirus Serotype 2 from Late Endosomes

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The internalization pathway and mechanism of uncoating of human rhinovirus serotype 2 (HRV2), a minor-group human rhinovirus, were investigated. Kinetic analysis revealed a late endosomal compartment as the site of capsid modification from D to C antigenicity. The conformational change as well as the infection was prevented by the specific V-ATPase inhibitor bafilomycin A1. A requirement for ATP was also demonstrated with purified endosomes in vitro. Capsid modifications occurred at a pH of 5.5 regardless of whether the virus was entrapped in isolated endosomes or free in solution. These findings suggest that the receptor is not directly involved in the structural modification of HRV2. Viral particles found in purified endosomes of infected cells were mostly devoid of RNA. This supports the hypothesis that uncoating of HRV2 occurs in intact endosomes rather than by a mechanism involving endosomal disruption with subsequent release of the RNA into the cytoplasm.

Three steps determine the early events in viral infection of a host cell: adsorption to the plasma membrane by binding to specific receptors, penetration, and subsequent uncoating of the genome. It is well established that many enveloped and nonenveloped viruses enter a cell via receptor-mediated endocytosis, with membrane penetration and uncoating taking place from endosomes (23, 46). Internalization of receptors and extracellular fluid is initiated by invagination of clathrin-coated and noncoated regions of the plasma membrane (21, 66). After pinching off, these coated and noncoated preendosomal vesicles reach the same early endosome compartment (22). In early endosomes, membrane proteins and the internalized material either are sorted into the recycling pathway or are directed via late endosomes to lysosomes for degradation (31). Moreover, endocytic routes of various plasma membrane proteins, ligands, toxins, and viruses have even been shown to converge with elements of the biosynthetic apparatus (15, 29, 55). Toxins and viruses are thereby transported to the respective compartment providing conditions suitable for delivery to the cytoplasm. The factors governing these different sorting mechanisms are largely unknown. At least, the low pH (6.5 to 5.0) prevailing in endocytic and exocytic compartments (49, 51) has been shown to be a prerequisite for translocation into the cytoplasm (46, 54, 55).

Although the entry pathway of many enveloped viruses is well characterized (23, 46), the early stages of infection of nonenveloped viruses remain obscure and controversial. In the case of picornaviruses, morphological as well as biochemical data have shown internalization of poliovirus to occur via coated pits and coated vesicles (45, 69, 71). However, the site of uncoating has not been clearly defined. Using cell fractionation, Kronenberger et al. (33, 34) have localized intact poliovirus sedimenting at 160S and modified 135S particles in lipid vesicles, which could not be identified as bona fide endosomes. Thus, they proposed a mechanism whereby 135S particles, which have lost VP4 but still contain RNA, are transferred across cellular organelles and are subsequently uncoated to 80S empty capsids in the cytoplasm (34). This is

contradicted by the most recent results of Perez and Carrasco (56), which favor uncoating in endosomes. Moreover, poliovirus degradation has not been detected up to 4 h after infection (7, 18, 68), arguing against an internalization pathway with final transport to lysosomes. This is again in contrast to the results of other groups, who have found poliovirus particles associated with lysosomal fractions (33, 34). Unlike poliovirus, human rhinovirus serotype 2 (HRV2), another member of the picornavirus family, seemed to be internalized by a clathrin-independent mechanism (45). Kinetic analysis of HRV2 entry by Lonberg-Holm and Korant (39) suggested an ultimate transport to and degradation in lysosomes, providing indirect evidence for uncoating along the endosomal pathway. Yet, the occurrence of native virions and modified HRV in endocytic organelles has not been demonstrated.

The influence of low pH on the picornavirus infective pathway has also long been under debate. Earlier work on poliovirus was in favor of an acid-dependent step (43, 44, 71), whereas recent results indicate that the low pH is not required for penetration and uncoating (18, 32, 56). In addition, chloroquine, an agent used to elevate the pH in acidic intracellular compartments, has been shown by Gromeier and Wetz (18) to exert a stabilizing effect on the capsid in vitro, whereas Kronenberger et al. (32, 34) have demonstrated that it promotes the formation of 80S empty capsids in vivo. Data on HRVs support the notion that the low intravesicular pH is necessary for successful infection to occur. This has been demonstrated for both major (56) and minor (52) receptor group viruses. Upon infection, structural alterations leading to a conversion of the 150S native infectious virions to 135 and 80S particles were observed (30); in contrast to the situation with poliovirus, similar particles could also be obtained by incubation of isolated HRVs in acidic buffers (26, 38, 40).

Receptor binding can also result in conformational transitions of viral capsids. In vitro, poliovirus forms 135 and 80S particles upon incubation with extracts of susceptible cells or soluble forms of the poliovirus receptor at a neutral pH above 30°C (6, 20, 27, 50). This again is in contradiction to in vivo data showing that intact poliovirus can be recovered from cells even 4 h after infection (7, 33, 69). Furthermore, viable mutants resistant to the receptor-induced inactivation have been obtained (28). The physiological significance of the receptor-mediated structural alteration is, therefore, still obscure. Binding of major receptor group HRVs to their soluble receptor,

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intercellular adhesion molecule 1 (ICAM-1), at a neutral pH also led to formation of 135 and 80S particles in various amounts, depending on the virus serotype (16, 26). For rhinoviruses of the minor receptor group the situation is even less clear. We have previously shown that the majority of HRV2 (a minor receptor group prototype) taken up by HeLa cells is structurally modified 30 min after infection; this modification is completely prevented by monensin, suggesting that in vivo the minor group receptor is not capable of catalyzing this transition when acidification is inhibited (52). Because of the lack of sufficient quantities of isolated minor-group HRV receptors, i.e., the low-density lipoprotein receptor (LDLR) and α_2 -macroglobulin receptor/LDLR-related protein, this virus-receptor interaction has not been studied so far in vitro (25).

The question of where and how picornaviruses in general and human rhinoviruses in particular deliver their genome to the cytosol is therefore still open. By using HRV2, the internalization pathway of a minor receptor group HRV was reinvestigated in order to determine the intracellular site and the mechanism of uncoating. Our results indicate that successful infection of HeLa cells requires a low-pH-dependent, but receptor-independent, uncoating step of HRV2 in late endosomes.

MATERIALS AND METHODS

Chemicals. Chemicals were obtained from Sigma. Bafilomycin A1, kindly provided by K. H. Altendorf, University of Osnabrück, Osnabrück, Germany, was dissolved in dimethyl sulfoxide at 20 mM and stored at -20°C . The final concentration of dimethyl sulfoxide was kept below 1%; dimethyl sulfoxide was also present in control samples. Fluorescein isothiocyanate (FITC)-transferrin was prepared as described elsewhere (59). [^{35}S]methionine was obtained from American Radiolabeled Chemicals; lyophilized *Staphylococcus aureus* cells (IgG-Sorb) were obtained from The Enzyme Center.

Cell culture. HeLa cells (Wisconsin strain; kindly provided by R. Rueckert, University of Wisconsin) were grown in monolayers in MEM-Eagle (Gibco) containing heat-inactivated 5% fetal calf serum and 5% calf serum; in suspension culture Joklik's MEM (Gibco) supplemented with 7% horse serum was used.

Preparation of A and B particles. HRV2 was propagated and labeled with [^{35}S]methionine in HeLa cells and purified as described elsewhere (63). A mixture of A and B particles was produced from purified HRV2 by mixing virus with an equal volume of 1 M sodium acetate buffer (pH 5.0). The mixture was left at room temperature for 20 min, chilled, and neutralized with 1 volume of 0.5 M Tris (19). B particles were prepared by heating purified virions in medium containing 10% serum to 56°C for 30 min (40).

Viral protein synthesis. Infection of HeLa cells with HRV2 was carried out in the absence or in the presence of the respective acidification inhibitor. HRV2 was added at a multiplicity of infection of about 500. After 20 min the cells were pelleted and resuspended in fresh methionine-free medium. Four hours postinfection 20 μCi of [^{35}S]methionine was added. Fifteen hours postinfection the cells were pelleted and lysed in 300 μl of radioimmunoprecipitation assay (RIPA) buffer (5), and the virus was immunoprecipitated with polyclonal antiserum and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Endosome labeling. Cells (2×10^5 to 2×10^7) were preincubated for 30 min at 34°C with infection medium (MEM-Eagle containing 2% fetal calf serum and 30 mM MgCl_2) in the absence or presence of the respective acidifica-

tion inhibitors. Thereafter, cells were resuspended in fresh medium, and ^{35}S -labeled HRV2 (2×10^4 to 2×10^6 cpm) was added. Alternatively, a pH-sensitive fluid-phase marker (FITC-dextran [molecular weight, 70,000], extensively dialyzed and used at a final concentration of 5 mg/ml) was added to the incubation medium. Cells were incubated by rotation in a water bath at 34°C for various periods. Cells were then rapidly cooled to 4°C and washed with phosphate-buffered saline (PBS) (Ca^{2+} and Mg^{2+} free) containing 10 mM EDTA to remove surface-bound virus (41). For transferrin internalization HeLa cells were washed with PBS and incubated with serum-free medium for 30 min to deplete endogenous transferrin. Cells were then allowed to internalize FITC-transferrin (20 $\mu\text{g}/\text{ml}$) for an additional 30 min at 34°C (59). Thereafter, cells were processed as above.

Sucrose gradient sedimentation analysis of viral particles in endosomes. A total of 2×10^7 cells were incubated with 10^6 cpm of [^{35}S]methionine-labeled HRV2 at 34°C in the absence or presence of 400 nM bafilomycin A1 for various periods. After preparation of an endosome-enriched Golgi fraction (see below), the endosomal pellet was resuspended in 200 μl of PBS and sonicated after addition of Nonidet P-40 and SDS (final concentrations, 0.5 and 0.3%, respectively). Samples were layered onto 4-ml gradients of 5-to-30% (wt/wt) sucrose in 1 M NaCl-0.5% bovine serum albumin-0.02 M Tris-HCl, pH 8.1 (38). After sedimentation for 50 min at $200,000 \times g$ in a Sorvall TST 60.4 rotor, 4-drop fractions were collected from the bottom. Twenty microliters of each fraction was applied onto a filter and counted in a Betaplate liquid scintillation counter (Pharmacia).

Immunoprecipitation of HRV2 from whole cells and endosomes. Immunocomplexes were formed by incubation of the antisera with lyophilized *S. aureus* cells in RIPA buffer as described elsewhere (52). The cell pellets and supernatants were processed separately. Cell pellets were lysed in 150 μl of RIPA buffer (5) for 20 min at 0°C , and cell debris was removed by centrifugation. Alternatively, endosomal fractions were dissolved in $5 \times$ RIPA buffer. First, C-antigenic particles were precipitated with monoclonal antibody (MAb) 2G2 (52). Second, native virus remaining in the supernatant of the first precipitation was recovered with a rabbit antiserum raised against HRV2. Both precipitates were washed once in RIPA buffer and then in PBS. Pellets were boiled in Laemmli sample buffer (35) and analyzed on SDS-12.5% polyacrylamide minigels. The gels were soaked in 1 M sodium salicylate for 30 min, dried, and exposed to X-ray film. Autoradiographs of dried gels were scanned by using an LKB Ultrascan XL densitometer, and the sum of the total peak areas of VP1, VP2, and VP3 precipitated with monoclonal and polyclonal antibodies was calculated.

Preparation of endosome-enriched Golgi fractions. Cells were homogenized in 4 volumes of 0.25 M sucrose in TEA buffer (10 mM triethanolamine-10 mM acetic acid-1 mM EDTA titrated with NaOH to pH 7.4) with a ball-bearing homogenizer (2), and a postnuclear supernatant was prepared by centrifugation at $1,000 \times g$ for 10 min. A gradient was formed by adjusting the postnuclear supernatant to 1.3 M sucrose in TEA buffer and overlaying it with 4 ml each of 1.1 and 0.25 M sucrose in TEA buffer (47). After centrifugation in an SW40 rotor (Beckman Instruments) for 1 h at 40,000 rpm, an endosome-enriched Golgi fraction was collected at the 0.25-1.1 M sucrose interface. This fraction was concentrated by centrifugation in an SW40 rotor for 1 h at 40,000 rpm onto 50 μl of a 1.1 M sucrose cushion. The pellet was resuspended in the sucrose cushion and adjusted to 0.25 M sucrose with TEA buffer.

Percoll density gradient centrifugation. ^{35}S -HRV2 and FITC-dextran were internalized for 10 min at 34°C . All subsequent steps were carried out at 4°C . After removal of surface-bound virus, cells were homogenized in 0.25 M sucrose in TEA buffer, and a postnuclear supernatant was prepared as described above. The postnuclear supernatant was layered on a 22.5% solution of Percoll in 0.25 M sucrose-TEA buffer and spun in a 50.2 Ti rotor (Beckman Instruments) for 30 min at 17,000 rpm. Thereafter, 500- μl fractions were collected from the bottom of the gradient and assayed for radioactivity, FITC-dextran, and β -hexosaminidase. β -Hexosaminidase activity was determined as described elsewhere (47).

Preparation of endosomes by free-flow electrophoresis. Endosomes were loaded with FITC-dextran and ^{35}S -HRV2 for 10 min, washed, and homogenized. A microsomal pellet was prepared by spinning the postnuclear supernatant onto a 2.5 M sucrose cushion in an SW40 rotor at 40,000 rpm for 1 h. The pellet was resuspended and adjusted to 1 mg of protein per ml in 0.25 M sucrose in TEA buffer. The sample was then subjected to gentle trypsin treatment by incubation with 3% 1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin/mg of protein for 5 min at 37°C . The reaction was stopped by adding a 10-fold excess of soybean trypsin inhibitor at 4°C . Thereafter, microsomes were injected (1 ml/h) into a Bender and Hobein free-flow electrophoresis apparatus Elphor Vap 22 at 130 mA and 1,300 V with 0.25 M sucrose in TEA buffer in the chamber (11, 47, 61). Ninety-two fractions were collected (3 ml per fraction per h) and assayed for protein (4), radioactivity, and fluorescence.

Cell-free acidification assays. Acidification assays of FITC-containing endosomes were carried out immediately after isolation, essentially as described previously (11, 12). Endosome-enriched Golgi fractions (5 to 10 μl containing 1 to 5 μg of protein) were equilibrated for 2 h at room temperature in 2 ml of acidification buffer (150 mM KCl-5 mM MgSO_4 -20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] titrated with tetramethylammonium hydroxide [TMA] to pH 7.4) and then transferred to an Aminco-Bowman spectrofluorometer. Fluorescence was continuously monitored at excitation and emission wavelengths of 485 and 515 nm, respectively. Acidification was initiated by addition of 10 μl K-ATP (pH 7.4; final concentration, 2.5 mM). pH gradients were dissipated by addition of 4 μl of nigericin from a 0.5 mM stock solution in ethanol (final concentration, 1 μM). A pH calibration curve was generated by incubation of FITC-labeled endosomes in KCl buffers of various pH values in the presence of nigericin overnight at 4°C .

Cell-free conversion of HRV2. Endosomes were loaded with about 2×10^6 cpm of ^{35}S -HRV2 in the absence or presence of 70 mM NH_4Cl for 20 min at 34°C . In some experiments NH_4Cl was present throughout all manipulations. After preparation of an endosome-enriched Golgi fraction, endosomes were concentrated by centrifugation and resuspended in 0.25 M sucrose in TEA buffer. When buffers A, D, and E were used for further incubation, NH_4Cl was also added to the resuspension buffer. A 200- μl endosome-enriched fraction was then incubated for 10 min at 34°C with 800 μl of buffer A (80 mM KCl, 70 mM NH_4Cl , 5 mM MgCl_2 , 20 mM HEPES-TMA [pH 7.4]), buffer B (150 mM KCl, 5 mM MgCl_2 , 20 mM HEPES-TMA [pH 7.4]), buffer C (150 mM KCl, 5 mM MgCl_2 , 20 mM HEPES-TMA [pH 7.4], 10 mM ATP), buffer D (70 mM NH_4Cl 80 mM Na-acetate [pH 6.0]), or buffer E (70 mM NH_4Cl , 80 mM Na-acetate [pH 5.5]). Following incubation, 200 μl of $5\times$ RIPA buffer was added, and the samples were processed for immunoprecipitation.

Data presentation. If not indicated otherwise, data of a

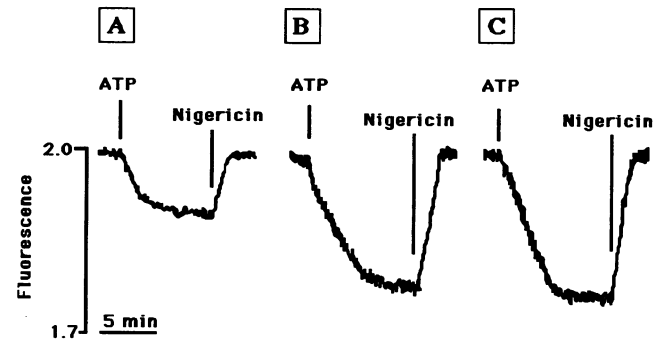


FIG. 1. Acidification properties of early and late HeLa cell endosomes. FITC-transferrin was internalized for 30 min at 34°C to label early endosomes (A). Late endosomes were labeled with FITC-dextran either by a 5-min pulse followed by a 10-min chase at 34°C (B) or by continuous internalization for 2 h at 20°C (C). After preparation of an endosome-enriched Golgi fraction, the corresponding labeled sample was equilibrated with acidification buffer (150 mM KCl, 5 mM MgSO_4 , 20 mM HEPES-TMA [pH 7.4]) at room temperature. pH-dependent fluorescence intensities were then recorded at excitation and emission wavelengths of 485 and 515 nm, respectively. ATP-generated pH gradients were dissipated by the K^+/H^+ ionophore nigericin as indicated by the rapid restoration of baseline FITC fluorescence.

single representative experiment are shown. All experiments were repeated three to five times with similar results.

RESULTS

In vitro acidification of HeLa cell endosome subcompartments. As a prerequisite for infection, HRV2 has to undergo a low-pH-dependent conformational change to C-antigenic particles (52); this takes place most likely in endosomes. Internalized ligands en route to lysosomes have been shown to gradually encounter a decreasingly lower pH in endosomal subcompartments, ranging from 6.5 to 5.0 (49, 60, 61, 70). Since this is not a general property of all cell types (62), the acidification of selectively labeled early and late endosomes was investigated in HeLa cells by an in vitro acidification assay (11, 13). Early recycling endosomes (1, 61, 64) were labeled with FITC-transferrin. Late endosomes (60, 61) were labeled with the fluid-phase marker FITC-dextran either by a pulse-chase (a 5-min pulse followed by a 10-min chase at 34°C) or under conditions known to block transport to lysosomes (continuous uptake for 2 h at 20°C) (17). After homogenization, endosome-enriched Golgi fractions were prepared by flotation in discontinuous sucrose density gradients (11, 12, 47). The respective endosome fractions were added to KCl-containing buffer, and the fluorescence was continuously recorded. A moderate drop in FITC fluorescence, indicative of intravesicular acidification, was seen for early endosomes after addition of ATP (Fig. 1A). A more pronounced ATP-dependent acidification was evident for both preparations of FITC-dextran-labeled late endosomes (Fig. 1B and C). In all cases, the K^+/H^+ ionophore nigericin completely dissipated the pH gradients. These experiments establish that, in HeLa cells, early and late endosomes indeed differ in their internal pH.

On the basis of pH calibration of the intravesicular dyes, the average pH in early endosomes was determined to be 6.9. For late endosomes a pH of 6.4 was found, regardless of whether the late endosomes were labeled by pulse-chase at 34°C or by incubation at 20°C (Fig. 1B and C). This indicates that internalization at or below 20°C indeed blocks transport

between late endosomes and lysosomes in this cell type. The same pH values have previously been found in CHO cells *in vitro* (12, 59, 60, 61). However, *in vivo* measurements have established pH values of ≤ 6.2 and ≤ 5.3 prevailing in early and late endosomes, respectively (60). On the basis of the identical pH values obtained *in vitro* for these two cell lines, there is reason to believe that the *in vivo* pH values in HeLa and CHO cell endosome subcompartments are also comparable.

Kinetics of virus entry and conversion to C-antigenic particles. As shown by Gruenberger et al. (19), maximal conformational change of isolated HRV2 is achieved by incubation in buffers with a pH lower than 5.6. Since the pH is gradually decreasing from early to late endosomes in HeLa cells (see above), the time course of the conformational change of HRV2 *in vivo* allows identification of the endosomal subcompartment in which this transition takes place. Cells were infected with ^{35}S -labeled HRV2, and the total cell-associated virus as well as altered particles was determined as a function of incubation time. The amount of C-antigenic particles was monitored by immunoprecipitation with MAb 2G2, which specifically recognizes an epitope appearing on the viral capsid upon exposure to a low-pH environment (52). Native virus was determined by immunoprecipitating the remainder with a polyclonal antiserum. Because of the substantially decreased affinity of HRV2 to its receptor at 0°C (39), in all experiments virus was continuously internalized for various periods at 34°C. Our results therefore represent steady-state values and thus permit direct comparison with data obtained by Lonberg-Holm and Korant (39). Under these conditions a linear increase of total cell-associated HRV2 up to 25 min was observed (Fig. 2A). Following a lag period of about 10 min, maximum conversion (40% of total cell-associated virus) to C-antigenic particles was also obtained at 25 min. On the basis of the kinetics of transport through early endosomes (half-life, 3 to 5 min [60, 61]), the conversion to C antigenicity is in accordance with arrival in late endosomes. Moreover, since the *in vivo* pH in early endosomes is clearly higher than 5.6, the threshold pH for conversion of isolated HRV2 (19), this strongly suggests that the presence of the minor-group receptor does not significantly facilitate the conformational change of HRV2.

After 25 min a rapid decrease of cell-associated immunoprecipitable radioactivity was observed. This coincides with the arrival of ligands in lysosomes (31, 61) and is in accordance with the data of Lonberg-Holm and Korant (39), who demonstrated trichloroacetic acid-soluble radioactivity after 20 min of infection. About half of the input virus is recovered from the supernatant in its C-antigenic form and represents eluted particles described by Lonberg-Holm and Korant (39). Since the time course of conversion of cell-associated virus and that of the appearance of C-antigenic virus in the supernatant are identical, eluted particles are most likely due to recycling and not to release of surface-bound particles as originally assumed.

Colocalization of HRV2 with a fluid-phase marker in isolated endosomes. The results presented above as well as our previous investigations have provided indirect evidence for a conformational change of HRV2 in endocytic compartments (52). To directly demonstrate the presence of HRV2 in endosomes, two different cell fractionation protocols were applied: (i) separation in self-forming Percoll density gradients and (ii) free-flow electrophoresis. ^{35}S -labeled HRV2 was internalized together with the fluid-phase marker FITC-dextran for 10 min at 34°C. After removal of plasma membrane-associated virus with EDTA (41), cells were homogenized with a ball-bearing homogenizer to ensure minimal rupture of intracellular compartments (2). A postnuclear supernatant was prepared and fractionated on a Percoll gradient. Under these

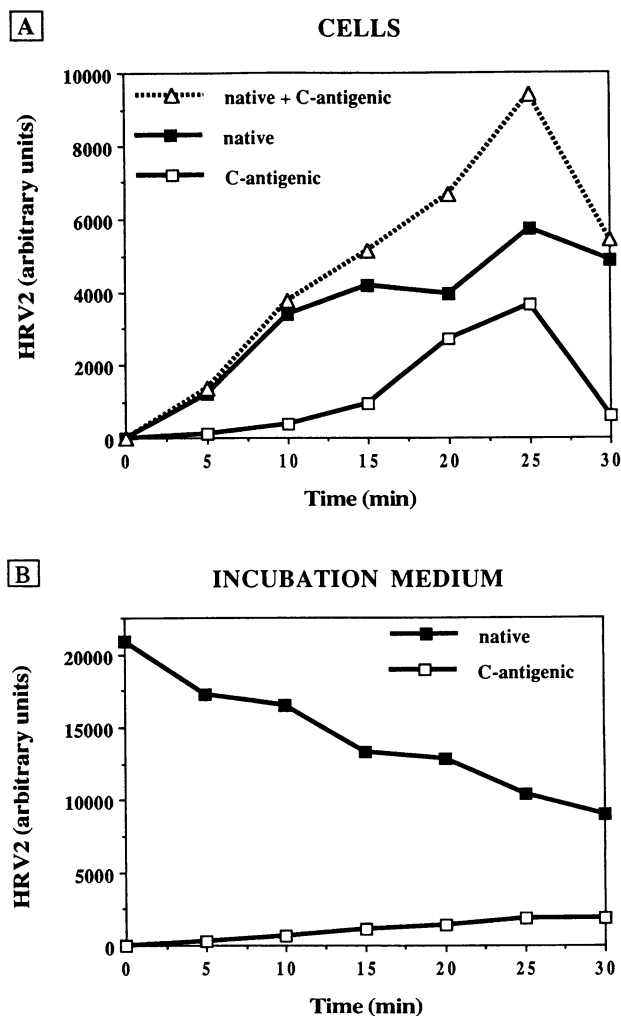


FIG. 2. Kinetics of virus entry into HeLa cells. Cells (10^6) were preincubated in 1 ml of infection medium for 30 min at 34°C and infected with 20,000 cpm of ^{35}S -labeled HRV2. After the periods indicated, the cells were pelleted and immediately resuspended in 10 ml of ice-cold PBS containing 10 mM EDTA to stop viral uptake and to remove cell surface-bound virus. The cells were lysed in RIPA buffer, and C-antigenic particles were immunoprecipitated from the lysed cells (A) as well as from the incubation medium (B) by immunoprecipitation with MAb 2G2. Remaining native virus was precipitated with polyclonal antiserum. The precipitates were analyzed by SDS-PAGE, autoradiographed, and quantitated by laser densitometry. Peak areas in arbitrary units are plotted. Total immunoprecipitated viral protein is also shown.

conditions plasma membranes, Golgi apparatus, endoplasmic reticulum, and endosomes are well resolved from high-density lysosomes (13, 47). As shown in Fig. 3A, HRV2 colocalizes with FITC-dextran-labeled endosomes in the low-density region of the Percoll gradient well separated from the lysosomal marker enzyme β -hexosaminidase. Since this gradient is ineffective at separating endosomes from other smooth membranes, the presence of HRV2 in endosomes was established by free-flow electrophoresis (11, 47, 61), which separates particles on the basis of differences in surface charge. HRV2 and FITC-dextran were internalized as described above; microsomes were prepared and subjected to free-flow electrophoresis. Under the conditions described by Marsh et al. (47),

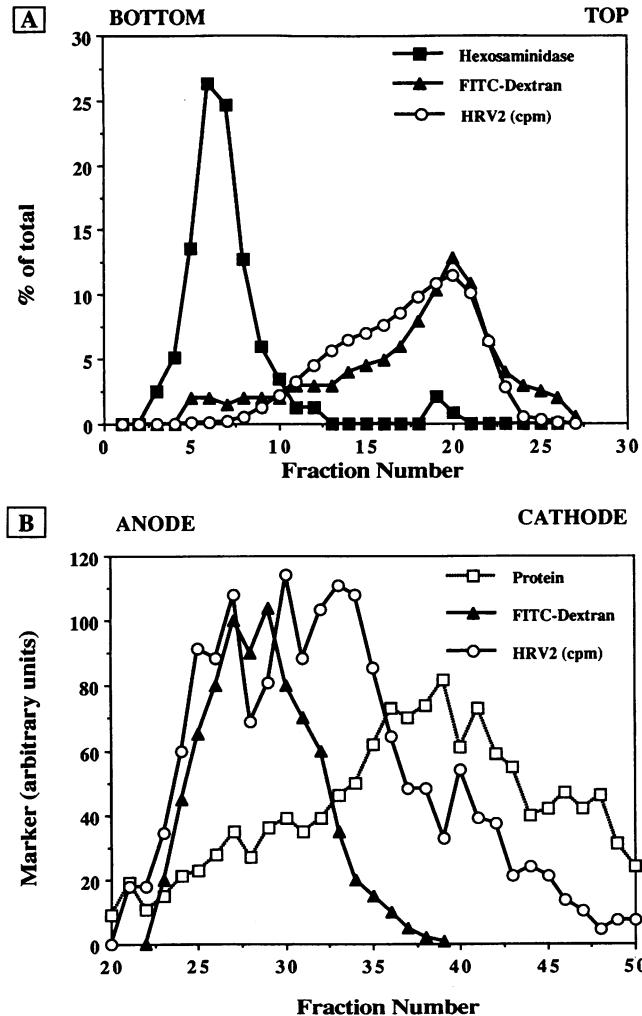


FIG. 3. Colocalization of HRV2 with FITC-dextran in isolated endosomes. ^{35}S -HRV2 (2×10^6 cpm) and FITC-dextran (5 mg/ml) were internalized into 2×10^7 HeLa cells for 10 min at 34°C . Plasma membrane-bound HRV2 was removed by EDTA treatment, cells were homogenized, and a postnuclear supernatant was prepared. (A) The postnuclear supernatant was then separated on a 22.5% self-forming Percoll density gradient. The lysosomal marker β -hexosaminidase in the high-density region is well separated from FITC-labeled endosomes in the low-density region of the gradient. In addition, HRV2 labeled vesicles colocalize with the fluid-phase marker FITC-dextran. (B) Microsomes were obtained by centrifugation of the postnuclear supernatant at $200,000 \times g$ for 1 h. After mild trypsin treatment microsomes were subjected to free-flow electrophoresis under the conditions described in Materials and Methods. FITC-dextran-labeled endosomes are shifted toward the anode, whereas the major protein peak is more deflected to the cathode. A substantial fraction of HRV2 labeled structures colocalize with FITC-dextran-containing endosomes; the remainder migrates at a position characteristic for the recycling compartment (65).

the major protein peak containing plasma membranes, endoplasmic reticulum, and Golgi apparatus is eluted undeflected whereas endosomes and lysosomes are shifted towards the anode (11, 61). Figure 3B demonstrates the colocalization of a major fraction of HRV2 with FITC-dextran in these anodally deflected endosomes. However, virus particles are also found at a position known to correspond to the recycling compart-

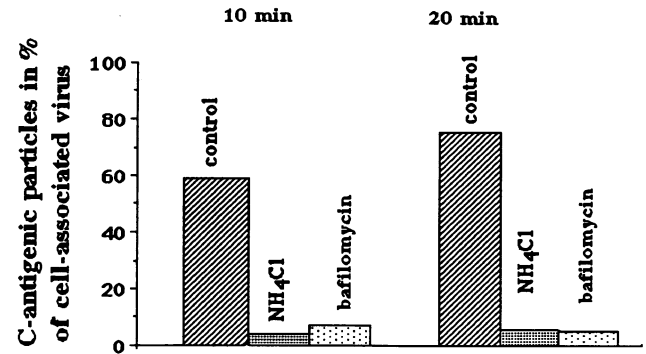


FIG. 4. Inhibition of the conformational change of HRV2 by bafilomycin A1 and ammonium chloride. Infection with ^{35}S -labeled HRV2 was carried out after 30 min of preincubation of the cells in infection medium in the absence (control) or in the presence of 400 nM bafilomycin A1 or 70 mM NH_4Cl . Ten and 20 min after infection at 34°C , the cells were lysed, C-antigenic particles were immunoprecipitated with MAb 2G2, and then the remaining native virus was immunoprecipitated. Immunoprecipitates were processed as for Fig. 2. C-antigenic virus + native virus = 100%.

ment (65). This is in good agreement with 50% of input virus being recycled as C-antigenic particles into the medium (Fig. 2B). Taken together, the results of these two separation techniques demonstrate the exclusive localization of HRV2 in endosomes.

Influence of the low endosomal pH on conformational change and infectivity. Having directly demonstrated the internalization of HRV2 into endosomes, we next reexamined the effect of the low endosomal pH on the conformational change of the viral capsid and on viral replication. Lysosomotropic agents such as ammonium chloride, chloroquin, and monensin have been shown to inhibit infection by HRV2 (43, 52). These substances exert, however, secondary effects which may not be related to elevation of the pH in acidic compartments (18, 32, 56). Endosomes, lysosomes, coated vesicles, and the Golgi apparatus (49, 51) have been shown to lower their internal pH via the activity of vacuolar-type proton pumps (V-ATPases). Therefore, we investigated the influence of the specific inhibitor of vacuolar-type proton pumps, bafilomycin A1 (3, 67), on early steps of infection. Cells were preincubated in the absence or presence of either bafilomycin A1 or NH_4Cl to dissipate any existing pH gradient. The conformational change of HRV2 after 10 and 20 min following infection in the presence of the respective compound was monitored by immunoprecipitation with MAb 2G2. While under control conditions about 60 to 70% of cell-associated virus was converted to the C-antigenic form, the conformational change was almost completely inhibited by 400 nM bafilomycin A1 at both time points (Fig. 4). The same effect was observed by internalizing the virus in the presence of 70 mM NH_4Cl . This unequivocally demonstrates that the activity of the endosomal proton pump is required for the conformational change of HRV2 to occur.

To test whether the low endosomal pH is also required for successful infection, the influence of bafilomycin A1 on viral infectivity was assayed. Cells were preincubated as described above and infected with HRV2 at a multiplicity of infection of 300. Subsequently, newly synthesized proteins were labeled with [^{35}S]methionine. After 15 h the production of viral progeny was determined by immunoprecipitation with polyclonal antiserum. As shown in Fig. 5A, lane -30, the continuous presence of the drug in the infection medium clearly

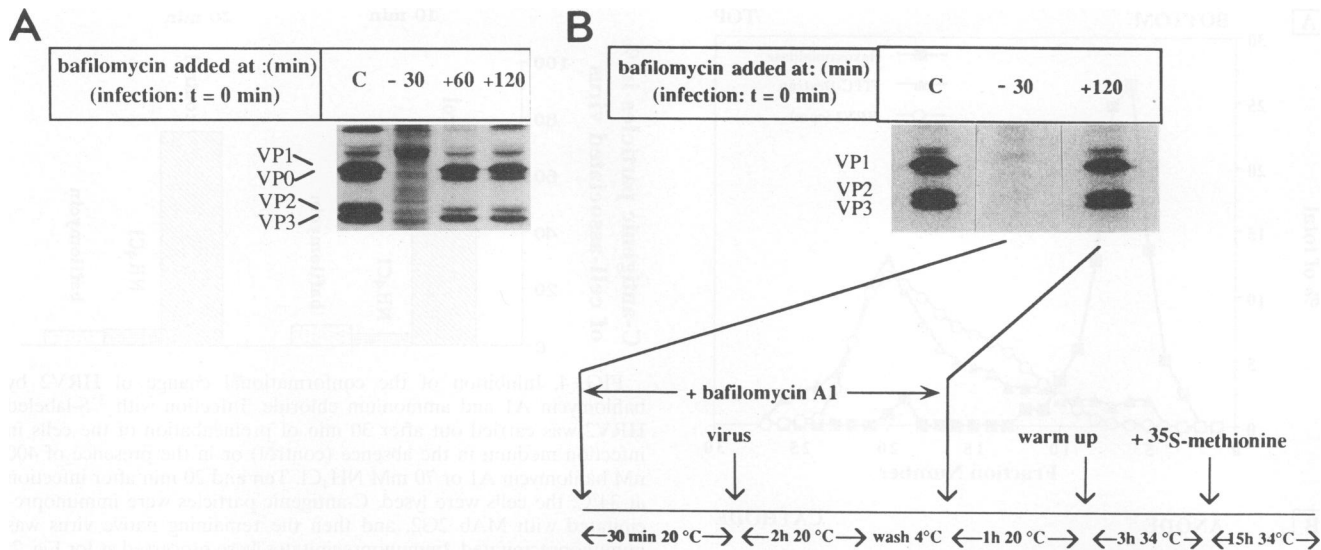


FIG. 5. Influence of bafilomycin A1 on viral reproduction. Cells (5×10^5) were preincubated in methionine-free infection medium for 30 min by slow rotation at 34°C (A) or at 20°C (B) and were infected with HRV2 (time t , 0 min). Bafilomycin A1 (400 nM) was added either before the infection (t , -30 min) or at 60 or 120 min postinfection. Newly synthesized proteins were labeled at 34°C with [35 S]methionine; progeny virus was immunoprecipitated with polyclonal antiserum, analyzed on SDS-PAGE, and autoradiographed. C, control (no addition of bafilomycin A1).

prevented viral infection. Its addition 1 (lane +60) or 2 (lane +120) h after viral challenge somewhat reduced the viral yield compared with the control (lane C), possibly by an unspecific effect on the protein synthesis machinery. This clearly shows that the conformational change induced by the endosomal V-ATPase is a prerequisite for viral uncoating. The inability of the drug to prevent infection when added 1 h postinfection is in good agreement with the rapid conversion of HRV2 to C-antigenic particles (Fig. 2A); it also demonstrates that uncoating is already completed after this period.

To strengthen the evidence for endosomal uncoating and/or penetration, cells were loaded with HRV2 at 20°C for 2 h, allowing for viral accumulation in late endosomes (see above). Under these conditions native virus is completely converted to C-antigenic particles (52). Bafilomycin A1 was then added, and after further incubation at 20°C the cells were shifted to 34°C to allow for protein synthesis. Under these conditions viral reproduction (Fig. 5B, lane +120) was indistinguishable from the control infection (lane C), whereas addition of bafilomycin prior to infection prevented viral reproduction (lane -30).

Analysis of viral particles in isolated endosomes. When native 150S virions are internalized, they are rapidly converted from D to C antigenicity (135S particles lacking VP4 and 80S particles which are also devoid of their genomic RNA [37, 39, 40, 42]). The intracellular site where this process takes place has never been determined with certainty. Since our results were highly suggestive of penetration or even uncoating of HRV2 from endosomes, we analyzed the nature of subviral particles in these organelles. To exclude the attachment of lipophilic, conformationally altered virions to vesicles during the fractionation procedure, at first a control experiment in which isolated 150, 135, and 80S particles were added to a postnuclear supernatant of HeLa cells and subjected to flotation in sucrose density gradients was carried out. Under these conditions, mostly endosomes and Golgi elements float up to the 0.25-1.1 M sucrose interface (11, 47); no added viral particles were found associated with this fraction, excluding nonspecific attachment of viral particles (data not shown). Next, HRV2 was internalized for 10 min at 34°C, and an

endosome-enriched fraction was prepared. Vesicles were disrupted with Nonidet P-40 and SDS, and viral particles were separated on linear sucrose density gradients (38). As presented in Fig. 6A, about 10% native virions, 57% 135S particles, and 23% 80S particles were found in endosomes. However, in the presence of bafilomycin A1, exclusively native virus with a sedimentation constant of 150S could be detected (data not shown).

When virus internalization was carried out for 2 h at 20°C, a condition in which virus accumulates in late endosomes (Fig. 1C) (17), all cell-associated virus was converted to C-antigenic particles (52). Examination of subviral particles recovered from endosomes under these conditions revealed 40% of 135S and 60% of 80S particles but no detectable native virions (Fig. 6B). Again, bafilomycin A1 completely inhibited the formation of C-antigenic particles (data not shown). These experiments demonstrate that HRV2 can efficiently uncoat and infect cells (Fig. 5B) (52) when accumulated in late endosomes. In contrast to major-group rhinoviruses (36, 39), the conformational change and uncoating of HRV2 can occur at 20°C. Taken together, our data strongly suggest that HRV2 is conformationally altered as well as uncoated in endosomes.

Establishment of an in vitro system: is the receptor required for conformational change and uncoating of HRV2? To investigate the requirements for a conformational change of HRV2 in isolated endosomes, 35 S-labeled virus was internalized in the absence or presence of 70 mM NH_4Cl (Fig. 4). This lysosomotropic agent completely prevents the transition to C-antigenic particles; in contrast to bafilomycin A1, which irreversibly inhibits V-ATPases, it can be completely removed. To prevent passive acidification (see below) NH_4Cl has to be present throughout the isolation procedure. After virus uptake, cells were homogenized and a postnuclear supernatant was subjected to separation on sucrose flotation gradients. About 60% of total HRV2 loaded onto the gradient could be recovered in an endosome-enriched Golgi fraction. No differences were found in recoveries and enrichment (about 80-fold with respect to the original homogenate) between control and NH_4Cl -treated cells. After incubation of isolated endosomes in various

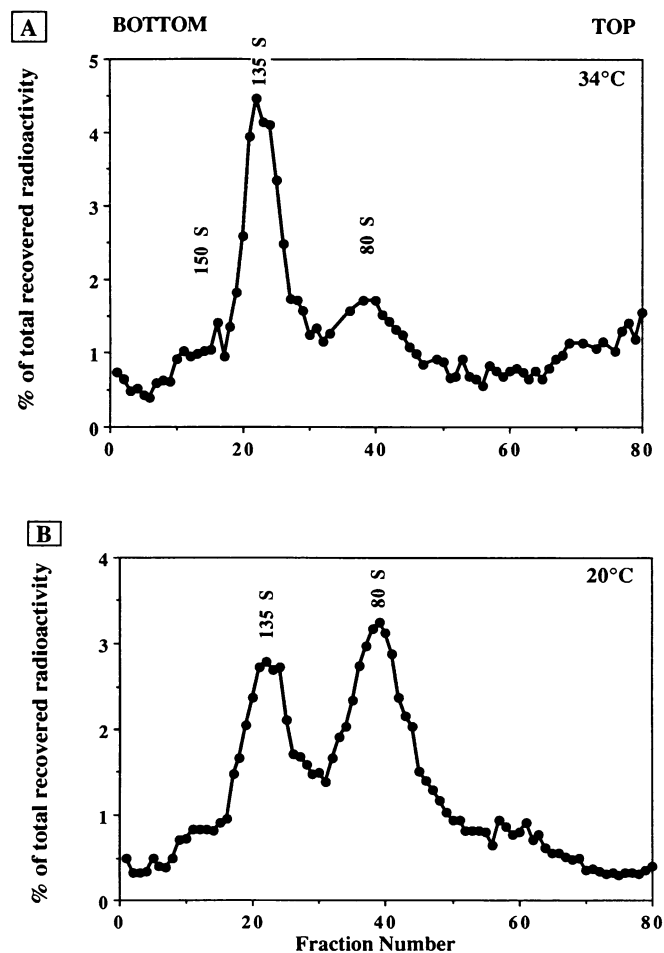


FIG. 6. Analysis of viral particles in isolated endosomes of infected cells. [³⁵S]methionine-labeled HRV2 was internalized into HeLa cells for 10 min at 34°C (A) or for 2 h at 20°C (B). Endosomes were disrupted, and the liberated viral particles were analyzed on linear sucrose gradients as described in Materials and Methods.

buffers (see Materials and Methods) the amount of C-antigenic particles and native virus was determined by immunoprecipitation with MAb 2G2 and then with polyclonal antiserum. Under control conditions (20-min internalization in the absence of NH₄Cl), 78% of C-antigenic particles were immunoprecipitated from isolated endosomes. As already shown for total cell-associated virus (Fig. 4), this conversion could be completely prevented by NH₄Cl (Fig. 7A). Therefore, all subsequent experiments were performed with virus internalized in the presence of NH₄Cl, and the conformational change was then induced *in vitro* in isolated endosomes. Three methods of endosome acidification were employed.

(i) **Artificial acidification.** Isolated endosomes were incubated in highly permeable buffers of various pH values. To exclude passive acidification, the NH₄Cl concentration was maintained at 70 mM (Fig. 7B).

(ii) **Passive acidification.** Dilution of NH₄Cl-loaded vesicles into NH₄Cl-free medium results in a rapid efflux of membrane-permeable NH₃ due to the concentration gradient. The remaining intravesicular protons dissipate more slowly and therefore lead to a transient decrease in pH (8) (Fig. 7C).

(iii) **Active acidification via the endosomal proton pump after complete removal of NH₄Cl and addition of ATP** (Fig.

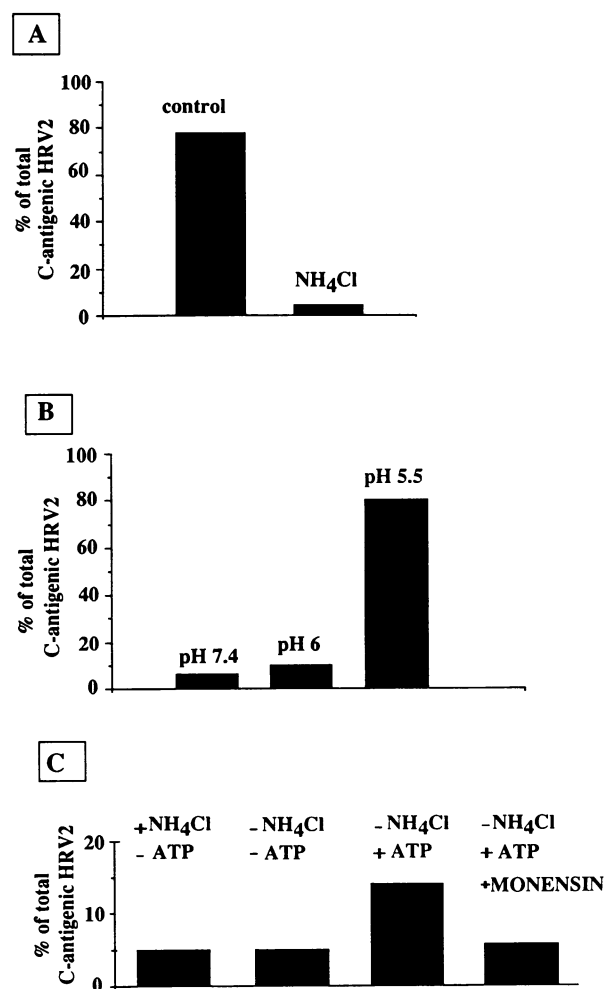


FIG. 7. Immunoprecipitation of HRV2 from isolated endosomes. [³⁵S]methionine-labeled HRV2 was internalized into HeLa cells for 20 min at 34°C in the absence or presence of 70 mM NH₄Cl, endosomes were prepared, and the amount of C-antigenic particles was determined and quantitated as for Fig. 4 (A). Prior to analysis the endosomes from ammonium chloride-treated cells were incubated with buffers of the indicated pH values (B). Endosomes as for panel B were incubated in acidification medium, pH 7.4 (Fig. 1), for 20 min at 34°C in the presence of the compounds indicated (C).

7C; also, Fig. 1). First we determined whether the minor group receptor can induce or facilitate the conformational transition to C-antigenic particles. It can be assumed that HRV2 is internalized together with its receptor into endosomes. Hence, this could result in a different pH dependence of structural conversion in the presence of the receptor within endosomes compared with isolated HRV2 (19). Incubation of virus within endosomes in a pH 6.0 or 7.4 medium did not result in generation of C antigenicity (Fig. 7B). However, maximal conformational change of 80% was observed by incubation of the endosome-entrapped virus in a pH 5.5 buffer. Thus the conformational change occurs at a pH of 5.5 regardless of whether the virus is trapped in endosomes or free in solution. Therefore, within the resolution of the experiment, the presence of the receptor does not seem to modulate the pH-dependent conformational change of HRV2.

As shown in Fig. 7C, mere dilution of NH₄Cl leading to

passive acidification did not suffice to induce a conformational change. In contrast, incubation in the presence of 10 mM ATP to activate the endosomal proton pump led to a conformational change of 15% of total endosome-associated virus. This effect was indeed due to intraendosomal acidification, since prior addition of monensin completely inhibited this conversion by blocking the establishment of a pH gradient (Fig. 7C).

Taken together, these *in vitro* experiments show that the conformational change to C-antigenic particles can be induced in isolated endosomes by activation of the endosomal proton ATPase. This demonstrates that at least a subpopulation of endosomes can indeed lower its internal pH *in vitro* sufficiently to cause conversion to the C-antigenic form. This *in vitro* system now enables us to determine the requirements for uncoating of nonenveloped viruses from isolated endosomes.

DISCUSSION

The overall entry pathway of the minor-group rhinovirus HRV2 has been investigated in HeLa cells. After receptor-mediated endocytosis, incoming native virions are modified after a lag period of about 10 min to C-antigenic particles. This modification is induced by the low pH (<5.6) established by the vacuolar proton pump in late endosomes. The minor-group receptor seems to have primarily a binding function, rather than mediating the conversion to C antigenicity and/or uncoating. This situation seems to be similar to that of foot-and-mouth disease virus, which can infect unsusceptible cells by utilizing the Fc receptor in the presence of virus-specific antibodies (48).

Once the virions are in late endosomes, 135S particles are formed, and the RNA is released. Uncoating of the genome and membrane penetration presumably occur synchronously by extrusion of the RNA through a pore in the membrane, leaving 80S particles in the endosomal lumen. Residual native virus as well as modified particles are then transferred to lysosomes, where they are rapidly degraded. The significance of this pathway is supported by the data obtained with the specific inhibitor of the endosomal proton pump, bafilomycin A1. Modification of 150S native virus to C-antigenic 135 and 80S particles is completely blocked by this drug. Moreover, as a consequence thereof, viral infection is prevented.

Receptor-independent conversion and/or uncoating. The first step in virus infection of a host cell is the binding to a specific receptor. It is clearly established for poliovirus and major-group rhinoviruses that their respective receptors catalyze capsid modifications *in vitro* at physiological pH and temperature (16, 26, 27). Recently we have shown that the LDLR and the α_2 -macroglobulin receptor/LDLR-related protein can function as receptors for minor-group HRVs (25). LDLR and α_2 -macroglobulin receptor/LDLR-related protein follow a well-characterized pathway in being recycled from early endosomes back to the plasma membrane. However, the interaction between these receptors and their corresponding HRVs has not been investigated so far. Our present data support the view that the receptors are primarily required for HRV uptake into endosomes and are not directly involved in the modification or uncoating *in vivo*.

(i) Transit through early endosomes occurs with a half time of 3 to 5 min (60, 61). However, no conformational change was observed during this time.

(ii) Kinetic analysis defined the late endosome (pH <5.3) as the compartment where the transition to C-antigenic particles occurs. Thus, the pH threshold for conversion during virus entry *in vivo* and that for conversion of isolated virus induced by incubation in low-pH buffers were indistinguishable. More-

over, the same pH threshold was found for virus conversion in isolated endosomes when assayed *in vitro*.

(iii) In the presence of the V-ATPase inhibitor bafilomycin A1, only unmodified virus was recovered from isolated endosomes after infection at 34°C as well as at 20°C.

(iv) Receptor- and/or low-pH-mediated modification of poliovirus and major group HRVs *in vivo* is highly temperature dependent, occurring only above 23°C (20, 36). However, HRV2 was completely converted to C antigenicity when internalized at 20°C, with the majority of the particles being devoid of RNA. Moreover, infection occurred after the temperature was shifted to 34°C after addition of bafilomycin.

Taken together, these data are in accordance with receptor-independent conversion as well as uncoating of HRV2.

Compartment of conversion and uncoating. The specific V-ATPase inhibitor bafilomycin A1 has proven very useful for unequivocal demonstration of low-pH dependence in the entry pathway of nonenveloped viruses. However, the exact intracellular site of picornavirus uncoating was still unclear, since various endocytic as well as exocytic organelles maintain a pH gradient to the surrounding cytoplasm (51). Therefore, a major aim of this study was to identify the compartment where the modification and uncoating of HRV2 takes place. For the first time, we demonstrate the presence of a picornavirus in highly purified isolated endosomes using free-flow electrophoresis. Moreover, the kinetics of HRV2 degradation was in agreement with further transport to lysosomes. This differs from the data on poliovirus, for which no association with typical endosomal fractions was found (33, 34) and no degradation was observed (7, 18, 68). As shown by inhibition studies with bafilomycin A1, the low pH established by the endosomal V-ATPase is the only determinant for HRV2 modification. On the basis of the kinetics of virus entry and conversion, this low-pH-induced conformational change occurs in late endosomes. Since HRV2 was able to infect cells when internalized at 20°C, a condition known to block transport between late endosomes and lysosomes, virus uncoating or penetration must take place from late endosomes.

Mechanism of membrane penetration and uncoating. Two mechanisms for membrane penetration and uncoating of non-enveloped viruses have been proposed.

(i) Membrane insertion of hydrophobic capsid proteins leads to destabilization of the endosomal membrane and subsequent disruption, thereby delivering modified virus particles to the cytosol. There, once penetrated, the modified virus is uncoated to empty capsids.

(ii) After the conformational transition, hydrophobic capsid protein(s) inserts into the endosomal membrane, eventually in concert with the respective receptor, and forms a pore or channel through which the viral genome is released into the cytosol and thereby uncoated.

In the case of poliovirus, Fricks and Hogle (10) have shown that the amino terminus of VP1 which is externalized during the conformational change can form an amphipathic helix able to insert into the endosomal membrane. This again could lead to either membrane destabilization or pore formation. VP1 of HRV2 seems to play a role in uncoating similar to that described for poliovirus: peptides derived from the N terminus of VP1 induced release of internalized macromolecules from isolated endosomes (57, 58). Recent mutational analysis has demonstrated that VP4 of poliovirus, the myristylated protein which is lost during the transition to 135S particles, could also play a role in the release of the RNA by insertion into the endosomal membrane (50).

Virus penetration by an endosome-disrupting mechanism should result in the preferential loss of those virions which

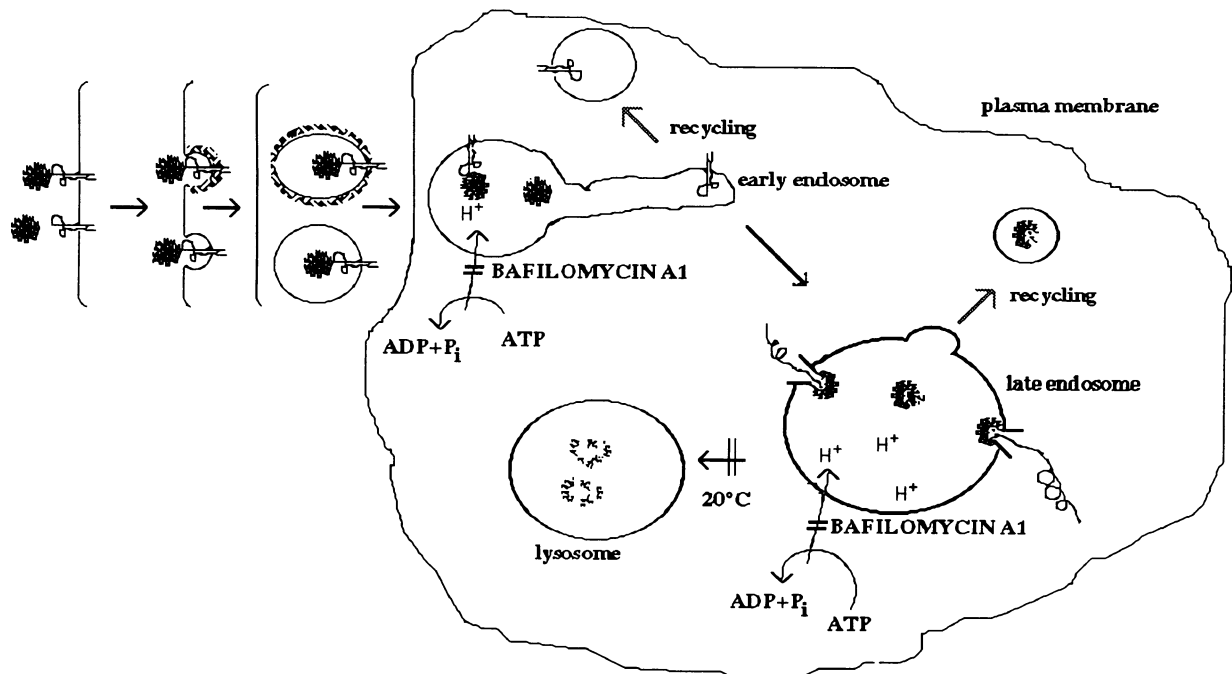


FIG. 8. Proposed mechanism of HRV2 entry and uncoating of the viral genome. After binding to its receptor, HRV2 is internalized via clathrin-coated or noncoated pits and vesicles into early endosomes. There, the viral receptor is sorted via recycling endosomes back to the plasma membrane, whereas HRV2 is transferred to late endosomes. Once in late endosomes, HRV2 undergoes acid-induced conformational changes of its capsid proteins. This leads to insertion of viral proteins into the endosomal membrane, whereby a pore through which the RNA is released into the cytosol is formed. Conformationally altered RNA-containing and RNA-free particles are either recycled into the external medium or degraded in lysosomes.

have undergone modification and membrane insertion and consequently induced endosome lysis. Therefore, few 135S particles and virtually no empty capsids should be present in isolated endosomes. We found 57% 135S and 23% 80S particles in isolated endosomes after 10 min of infection at 34°C and up to 40% 135S and 60% 80S particles after 2 h of internalization at 20°C. These data are in favor of a pore-forming rather than an endosome-disrupting mechanism. Adenovirus, which penetrates via endosome disruption (9, 14), is not degraded in lysosomes. In contrast, lysosomal degradation of HRV2 was clearly evident. Moreover, pore formation is also supported by our recent *in vitro* experiments, in which release of macromolecules of different molecular weights, cointernalized with HRV2 or adenovirus, was investigated. Virus-induced release of these markers was size independent for adenovirus but was dependent on the molecular weight for HRV2 (57, 58). Therefore, we propose that HRV2 releases its RNA via a specific pore without disrupting the endosome. A similar mechanism has also been proposed by Perez and Carrasco (56) to describe the poliovirus-mediated, bafilomycin-inhibitable release of α -sarcin into the cytoplasm. However, poliovirus uncoating per se is independent of low intravesicular pH.

Our data suggest the following concept for the early events of HRV2 infection (Fig. 8). After binding and internalization into early endosomes, the virus-receptor complex is dissociated, probably as a result of the mildly acidic pH environment. While the receptor is recycled, HRV2 is transferred to late endosomes, where it is structurally modified by pH < 5.6, established by the endosomal proton pump. Most likely, this results in membrane insertion of VP1, thereby allowing translocation of the viral RNA via a pore into the cytoplasm.

This raises the question of the mode of rhinovirus RNA translocation across the endosomal membrane and the driving forces underlying this process. Madshus et al. (45) provided very indirect evidence that the membrane potential might play a role in uncoating of HRV2, but not of poliovirus, since plasma membrane depolarization led to enhancement of infection for rhinovirus only. However, their experimental design did not allow differentiation of membrane potential effects on virus entry, conformational change, uncoating, or viral replication. In addition, the potential difference between endosomes and the cytoplasm is unknown. The plasma membrane maintains a transmembrane potential of about 40 mV, cell interior negative. Subcellular distribution of potential sensitive dyes in *Dictyostelium* amoebae suggests that acidic intracellular compartments are inside negative with respect to the cytoplasm (24). This would pose a driving force of negatively charged molecules such as RNA out of the endosome. Using our *in vitro* system, we are currently testing an assay to measure RNA release from isolated endosomes to further investigate the mechanism and driving force of HRV2 uncoating.

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