Inhibition of Clathrin-dependent Endocytosis Has Multiple Effects on Human Rhinovirus Serotype 2 Cell Entry*

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Minor group human rhinoviruses (exemplified by human rhinovirus serotype 2 (HRV2)) use members of the low density lipoprotein receptor family for cell entry; all these receptors possess clathrin-coated pit localization signals. Viral infection should thus be inhibited under conditions of impaired clathrin-mediated endocytosis. However, Madshus et al. reported an increase in the cytopathic effect of HRV2 infection in HEp-2 cells upon suppression of clathrin-dependent endocytosis by hypotonic shock and potassium depletion (Madshus, I. H., Sandvig, K., Olsnes, S., and van Deurs, B. (1987) J. Cell. Physiol. 131, 14–22.) To resolve this apparent contradiction, we investigated the binding, internalization, conformational change, and productive uncoating of HRV2 in HeLa cells subjected to hypotonic shock and potassium depletion. This treatment led to an increase in HRV2 binding, with internalization being barely affected. The generation of C-antigenic particles requiring pH \( \leq 5.6 \) was strongly reduced due to an elevation of the pH in endosomal compartments. However, K\(^+\) depletion only slightly affected de novo viral protein synthesis, suggesting that productivity of viral RNA in the cytoplasm is enhanced and thus compensates for the reduction in C-antigenic particles. The distinct steps in the entry pathway of HRV2 are thus differentially influenced by potassium depletion. Viral internalization under conditions of inhibited clathrin-dependent endocytosis without the need to disturb the ionic milieu was confirmed in HeLa cells overexpressing the nonfunctional dynamin-1 mutant K44A. Unexpectedly, overexpression of dynamin-1 K44A resulted in elevated endosomal pH compared with overexpression of wild-type dynamin.

About 50% of all mild infections of the upper respiratory tract, generally recognized as common colds, are caused by human rhinoviruses. These small RNA-containing viruses constitute a large genus within the family Picornaviridae. The >100 serotypes are divided into two groups based on their receptor specificity (1, 2); major group viruses (91 serotypes) have access to the host cell by binding to intercellular adhesion molecule-1 (ICAM-1/CD54)\(^1\) (3–5), whereas minor group viruses (10 serotypes) bind to members of the low density lipoprotein receptor (LDLR) family (6, 7). Internalization into bona fide endosomes has been demonstrated for the minor group virus HRV2 and the major group virus HRV14 (8). All receptors of the LDLR family, including LDLR, LDLR-related protein (LRP), and very low density lipoprotein receptor (VLDLR), contain tyrosine- and dileucine-based internalization signals in their cytoplasmic tails that direct them into clathrin-coated pits (9). At least for LDLR, it has been explicitly shown that ligand internalization depends on functional clathrin coats (10). Although specific binding of HRV2 to these receptors is well characterized (6, 7, 11, 12), it is so far unknown whether any of them is preferentially used by HRV2 for productive infection of HeLa cells.

Soon after cell entry, native infectious viral particles undergo a conformational change induced by the low pH (<5.6) prevailing in endocytic carrier vesicles and early endosomes (13, 14). These modifications account for the formation of subviral “A-particles” sedimenting at 135 S that still contain the genomic viral RNA. Release of the RNA from the protein shell creates subviral “B-particles” that sediment at 80 S. Both particles are detected early in infection and are probably intermediates (A-particles) and remainders (B-particles) of the uncoating process (15). Whereas the native virus is “D-antigenic,” the subviral particles expose other epitopes and are “C-antigenic” (16). We have shown that inhibition of endosomal acidification by the specific vacuolar ATPase inhibitor bafilomycin A1 completely blocks the conformational modification from D- to C-antigenicity and prevents viral infection by HRV2 (14). As demonstrated by size-selective release of fluid-phase markers, which were co-internalized with the virus into endocytic compartments, pores are opened in response to the low pH in the presence of the virus (17). This suggests that the viral RNA enters the cytosol through a pore in the endosomal membrane. The acid-induced conformational change thus either precedes or is directly coupled to the transfer of the RNA through the endosomal membrane into the cytosol. Modified subviral particles are in part digested in lysosomes and in part recycled to the cell surface and can therefore be found in the cell supernatant early.

\(^1\) The abbreviations used are: ICAM-1, intercellular adhesion molecule-1; LDLR, low density lipoprotein receptor; LRP, low density lipoprotein receptor-related protein; VLDLR, very low density lipoprotein receptor; β-VLDL, β-very low density lipoprotein; HRV2, human rhinovirus serotype 2; HRV14, human rhinovirus serotype 14; GST, glutathione S-transferase; RAP, receptor-associated protein; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; HA, hemaglutinin; DMEM, Dulbecco’s modified Eagle’s medium; RIPA, radioligand precipitation assay; MEM, minimal essential medium; PFS, fetal calf serum; dynamin\(^{K44A}\), dynamin-1 mutant K44A; dynamin\(^{**}\), wild-type dynamin-1; MES, 2-(N-morpholino)ethanesulfonic acid; mAb, monoclonal antibody; m.o.i., multiplicity of infection.
after infection. Upon arrival of the genomic RNA in the cytosol, synthesis of new virus is initiated.

Although the endocytic subcompartment where HRV2 uncoating takes place has been characterized, the initial pathway of HRV2 entry is not completely resolved. All receptors that have been demonstrated to mediate HRV2 attachment contain clathrin localization signals, and viral infection via clathrin-dependent endocytosis would appear most likely. However, in 1987, when the nature of the minor group rhinovirus receptor was unknown, Madshus et al. (18) reported that inhibition of clathrin-dependent endocytosis by K⁺ depletion results in an increase in the cytopathic effect of HRV2 in human epidermoid HeP-2 cells, a finding that is strongly suggestive of clathrin-independent viral internalization.

HeLa cells stably transfected with a dominant-negative mutant of dynamin-1 (dynaminK444A) have been generated (19). The mutation results in loss of the GTPase activity, inhibition of clathrin-coated pit constriction, and arrest of clathrin-dependent endocytosis. Using these cells, it was recently shown that productive infection by the major group virus HRV14 is dependent on functional dynamin (20), although the receptor for the major group of human rhinoviruses, ICAM-1, lacks internalization signals (21). We used this genetic defect in dynamin to exclude nonspecific ion concentration effects as occur under K⁺ depletion to obtain internalization data for HRV2.

These seemingly irreconcilable data on the entry of rhinoviruses and, in particular, on that of the minor group virus HRV2 prompted us to investigate in detail the early events in viral infection. In particular, we dissected the effect of hypotonic shock/K⁺ depletion on the binding, internalization, conformational changes, and productive uncoating of HRV2. The results thereof, together with data obtained with cells expressing mutant dynamin-1, demonstrate that viral internalization can occur via LRPs and/or VLDLR by a clathrin-independent pathway.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All chemicals were obtained from Sigma unless specified otherwise. Bafilomycin A₁, kindly provided by Dr. K. H. Altendorf (University of Osnabrück, Osnabrück, Germany), was dissolved in Me₂SO at 20 mM and stored at −20 °C. The final concentration of Me₂SO (which was also added to the control samples) was kept below 1%. GST-RAP was prepared as described elsewhere (22). Conjugation of transferrin with fluorescein was carried out as described (23). FITC-dextran (70 kDa) was extensively dialyzed against Tris-buffered saline (pH 7.4) and finally against phosphate-buffered saline (PBS) before use.

Cy5.18-OSu (Cy5) was obtained from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom) and coupled to dextran (70 kDa) as described (24). Alexa 488-labeled goat anti-mouse antibody was purchased from Molecular Probes, Inc. (Eugene, OR); rhodamine-labeled mouse anti-hemagglutinin (HA) monoclonal antibody was obtained from Roche Molecular Biochemicals. Texas Red-conjugated transferrin was dissolved in PBS (5 mg/ml) and stored at −20 °C. Moviol 4–88 was purchased from Calbiochem and used at 10% in water. [35S]Cysteine/methionine (Tran 35S-label) and Na 125I were obtained from New England Nuclear (Wisconsin strain, a subclone selected for increased capacity to replicate human rhinoviruses) were incubated in serum-free Leibovitz L-15 medium for 30 min at 37 °C and K⁺-depleted as described (10). Briefly, cells were incubated in hypotonic medium for 5 min at 37 °C, followed by incubation in isotonic KCl-free buffer for 30 min at 37 °C. For control incubations, cells were maintained in Leibovitz L-15 medium as indicated above, and all incubations were carried out in isotonic KCl buffer.

FITC-Transferrin Binding and Internalization—Control or K⁺-depleted HeLa cells were cooled to 4 °C and incubated with 50 μg/ml FITC-transferrin in K⁺-containing or K⁺-free buffer for 20 min at 4 °C. Cells were washed with PBS²⁻ and lysed in 0.5 ml of lysis buffer, and cell debris were removed by centrifugation. The FITC fluorescence in the supernatant was measured in a CytoFluor 2300 (Millipore Corp.) using a standard filter set (excitation, 485 nm (20-nm slit width); emission, 530 nm (25-nm slit width)). After subtraction of the background fluorescence (unlabeled cells), the amount of cell-associated cell-associated transferrin was calculated using a calibration curve derived from serial dilutions of FITC-transferrin in lysis buffer.

To determine the internalization of plasma membrane-bound transferrin, an aliquot of the cells was washed with PBS²⁻ and incubated in the respective K⁺-containing or K⁺-free buffer at 37 °C for 20 min. To halt internalization, cells were again cooled, and membrane-bound transferrin was removed by washing the cells with 25 mM acetic acid containing 150 mM NaCl for 15 min at 4 °C, followed by washing with ice-cold PBS²⁻ for 15 min. This treatment completely removes plasma membrane-bound transferrin (23). After cell lysis, internalized transferrin was determined as described above.

β-VLDL Internalization—Control or K⁺-depleted HeLa cells were incubated in the respective K⁺-containing or K⁺-free buffers containing 10 μg/ml 125I-labeled β-VLDL for 20 min at 37 °C. Cells were cooled to 4 °C and washed with PBS²⁻ and lysed in 0.5 ml of lysis buffer, and cell debris were removed by centrifugation. The FITC fluorescence in the supernatant was measured in a CytoFluor 2300 (Millipore Corp.) using a standard filter set (excitation, 485 nm (20-nm slit width); emission, 530 nm (25-nm slit width)). After subtraction of the background fluorescence (unlabeled cells), the amount of cell-associated cell-associated β-VLDL was calculated using a calibration curve derived from serial dilutions of FITC-β-VLDL in lysis buffer.

**Fluid-phase Uptake**—Control or K⁺-depleted cells were incubated with 5 mg/ml FITC-labeled dextran (70 kDa) dissolved in K⁺-containing or K⁺-free buffer, respectively, for 20 min at 37 °C. Excess FITC-dextran was removed by washing the cells three times with ice-cold PBS²⁻. Cells were washed with PBS²⁻ and lysed in 0.5 ml of lysis buffer, and cell debris were removed by centrifugation. The FITC fluorescence in the supernatant was measured as described for FITC-transferrin.

**Viral Binding and Internalization**—To determine viral binding, control or K⁺-depleted cells grown in 12-well plates were incubated in isotonic K⁺-containing or K⁺-free buffer, respectively, in the presence of ~25 × 10⁳ cpm/well 35S-labeled HRV2 for 1 h at 4 °C. Cells were then washed with the same ice-cold buffers, and cell-associated radioactivity was determined as described above. Non-specific binding was determined by incubation in the respective buffer in the presence of 10 mM NaEDTA (pH 7.4) for 1 h at 4 °C.

The efficiency of removal of HRV2 bound to the cell membrane at 4 °C was assessed using the following methods: (i) extensive washing with K⁺-free buffer without CaCl₂ and MgCl₂, but supplemented with 10 mM NaEDTA; (ii) incubation with 20 mM MES titrated with tetra-methylammonium hydroxide to pH 5.0, 10 mM KCl, 140 mM NaCl, 1 mM CaCl₂ and 1 mM MgCl₂, 1 mg/ml glucose, and 0.5% bovine serum albumin; and (iii) incubation with 0.1% SDS, and 1% Triton X-100 (27).

**Cell Culture and Viral Propagation**—HeLa cells (Wisconsin strain, kindly provided by R. Rueckert, University of Wisconsin) were grown as monolayers in Eagle’s minimal essential medium (MEM) (Life Technologies, Inc.) containing heat-inactivated 10% fetal calf serum (FCS), 2 mM glutamine, and 1% penicillin/streptomycin culture in Joklik’s DMEM (Life Technologies, Inc.) supplemented with heat-inactivated 10% FCS, 400 μg/ml G418 (Life Technologies, Inc.), 200 ng/ml puromycin, and 1 μg/ml tetracycline. Cells were dislodged from subconfluent cultures with trypsin/EDTA, and 1.5 or 3.5 × 10⁵ cells/well were plated on 12- or 6-well plates (Falcon), respectively, in the absence of tetracycline for 48 h (induction) before use; at that time, the cells finally were 60% confluent. HRV2 was propagated, labeled with [35S]Cysteine/methionine, and purified as described (28).

K⁺ Depletion in HeLa Cells Following Hypotonic Shock—HeLa cells (Wisconsin strain, a subclone selected for increased capacity to replicate human rhinoviruses) were incubated in serum-free Leibovitz L-15 medium for 30 min at 37 °C and K⁺-depleted as described (10). Briefly, cells were incubated in hypotonic medium for 5 min at 37 °C, followed by incubation in isotonic KCl-free buffer for 30 min at 37 °C. For control incubations, cells were maintained in Leibovitz L-15 medium as indicated above, and all incubations were carried out in isotonic KCl buffer.

**HRV2 Entry**

3953

VDL
CaCl₂, 1 mM MgCl₂, and 1 mg/ml glucose for 2 × 5 min, followed by washing with ice-cold PBS²⁺; (iii) incubation with 2.5% trypsin (100 µl/well) for 60 min (as cells were detached from the plastic dishes by the latter treatment, they were collected and washed with PBS²⁺ by low speed centrifugation); and (iv) determination of 35S-labeled HRV2 remaining on the cell surface after immunoprecipitation by incubation with the virus-specific monoclonal antibody (mAb) 8F5 (29). For Method iv, cells were incubated with 200 µl/well 8F5 hybridoma supernatant for 2 h, washed with PBS³⁺, and lysed in RIPA buffer (500 µl/well). Virus-antibody complexes were then precipitated by addition of 30 µl of a 10% (v/v) IgG-sorb suspension for 3 h at room temperature. Immunocomplexes were pelleted at 13,000 × g for 5 min. One-hundred µl of 8F5 was then added to the supernatant; and incubation, immunocomplex formation, and pelleting were repeated. Pellets were washed twice with RIPA buffer and twice with PBS and suspended in 10 volumes of scintillation mixture (Ready-Value, Beckman Instruments) for counting. Finally, for viral uptake experiments, control or K⁻/-depleted cells were incubated with 25 × 10⁶ cpm ³⁵S-labeled HRV2 for 20 min at 34 °C in the respective buffer; cells were washed with ice-cold PBS containing 10 mM EDTA and pelleted; and cell-associated radioactivity was determined in the cell lysates as described above. 

**Immunoprecipitation of HRV2—S. aureus mAb 2G2 and S. aureus rabbit anti-HRV2 antisierum immunocomplexes were made as described (30). After incubation of the cells with HRV2, cell supernatants and pellets were pelleted separately. Pellets were washed with cold RIPA buffer for 15 min at 4 °C. Cell debris were removed, and D-antigenic particles were recovered by incubation with the mAb 2G2 immunocomplexes. D-antigenic particles remaining in the supernatant were then precipitated with rabbit antisierum against HRV2 and S. aureus cells. Precipitates were washed twice with RIPA buffer and twice with PBS and then analyzed on SDS-12% polyacrylamide minigels. Gels were soaked in 1M sodium salicylate for 30 min, dried, and exposed to x-ray film. Quantification was by laser densitometry (Ultrascan XL, Amersham Pharmacia Biotech) of the fluorographs.**

**Restoration of Cellular Protein Synthesis upon Hypotonic Shock and K⁻ Depletion—**All incubations were carried out at 34 °C. Control or K⁻/-depleted HeLa cells (1 × 10⁶ cells/ml for each time point) were incubated in isotonic K⁻-containing or K⁻/-free buffer, respectively. After 20 min, cells were resuspended in the respective buffers containing 200 mM bafilomycin A₁ to dissipate the low intravesicular pH. After 40 min, cells were transferred to methionine-free MEM with 2% FCS (containing bafilomycin A₁) and incubated further for 4, 6, 8, and 17 h. Cellular proteins were labeled with 1 µCi/ml [³⁵S]methionine for 1 h (10). Cells were washed with ice-cold PBS, and incorporated [³⁵S]methionine was measured after protein precipitation with 10% trichloroacetic acid. Protein synthesis in K⁻/-depleted cells at a given time point was related to that in the control cells, which was set to 100%. The efficiency of K⁻ depletion was always verified by quantification of FITC-transferrin internalization (see above).

**Viral Protein Synthesis—**All incubations were at 34 °C. Infection of control or K⁻/-depleted HeLa cells (1 × 10⁶ cells/ml) was carried out in K⁻/-free buffer, respectively, for each time point. HRV2 was added at a multiplicity of infection (m.o.i.) of 100 for 20 min. To halt uncouping of input virus, cells were transferred to fresh buffers supplemented with 200 mM bafilomycin A₁ and further incubated for 40 min. The drug was also present during all subsequent incubations. To allow for recovery of protein synthesis, which is reduced under K⁻/-depleted conditions, control and K⁻/-depleted cells were transferred to methionine-free MEM with heat-inactivated 2% FCS. Seven hours post-infection, the cells were pelleted and lysed in 300 µl of RIPA buffer. Virus was immunoprecipitated with anti-HRV2 antisierum and analyzed by SDS-polyacrylamide gel electrophoresis. The intensity of the viral protein bands was determined from fluorographs by laser densitometry.

**Fluorescence Microscopy—**HeLa cells expressing HA-tagged dynamin²⁴⁴⁺ or dynamin²⁴⁺ were plated at low density on 8-well chamber glass slides and cultivated in tetracycline-free DMEM for 48 h prior to the experiment. Inhibition of transferrin internalization into dynamin²⁴⁺ cells was verified by fluorescence microscopy. Cells were deprived of transferrin by incubation in serum-free DMEM for 30 min at 37 °C. Then they were resuspended in fresh medium containing 50 µg/ml FITC-transferrin for 20 min at 37 °C and cooled. Plasma membrane-bound transferrin was removed by acid wash (see above), followed by a wash with PBS, fixed with 4% paraformaldehyde for 1 h at room temperature, quenched with 50 mM NH₄Cl, permeabilized with 0.05% saponin, and incubated with rhodamine-labeled anti-HA monoclonal antibody (1:80 dilution). For HRV2 internalization, cells were then incubated with HRV2 at a m.o.i. of 100 in DMEM for 20 min at 34 °C. This high m.o.i. was found to be necessary for HRV2 detection. To study the influence of GST-RAP on HRV2 internalization, cells were preincubated in DMEM containing GST-RAP (100 µg/ml) at 4 °C for 1 h. GST-RAP was also present during HRV2 internalization. Cells were then transferred to 4 °C and washed with ice-cold PBS²⁺, and binding sites were blocked by incubating the cells with rabbit anti-HRV2 antisierum (preadsorbed with non-infected cells and diluted 1:10) for 1 h at 4 °C. Cells were washed with PBS²⁺, fixed with 4% paraformaldehyde in PBS at 1 h room temperature, quenched with 50 mM NH₄Cl, and permeabilized with 0.05% saponin. First, internalized D- and C-antigenic HRV2 was detected by indirect immunofluorescence using mAb 8F5 at 6 µg/ml and Alexa 488-labeled goat anti-mouse antibody (1:500). Then, cells overexpressing HA-dynamin²⁴⁺ and HA-dynamin²⁴⁺ were identified using a rhodamine-labeled monoclonal antibody specific for the HA epitope. It should be mentioned that a punctate fluorescence appeared in cells devoid of HA-dynamin expression, but with internalized HRV2. This resulted from interaction of the mouse anti-HA monoclonal antibody with residual binding sites present on the secondary anti-mouse antibody used to detect mAb 8F5 attachment to HRV2. Various attempts to block these free binding sites failed, but the appearance of the staining pattern allowed a clear differentiation. Cells were mounted in Moviol and viewed with a Zeiss Axioskop 2 microscope. Digital images were processed with the Zeiss KS400 imaging program. Confocal images were taken with a Leica TCS NT Universal confocal microscope.

**Determination of the Fraction of Dynamin²⁴⁺⁺-expressing Cells—**Impaired clathrin-dependent internalization directly correlates with the number of transferrin receptors at the plasma membrane. The percentage of dynamin²⁴⁺⁺-expressing cells was thus determined via fluorescence-activated cell sorting analysis of FITC-transferrin binding (31). Induced cells were incubated for 30 min in serum-free DMEM, cooled to 4 °C, and incubated with 50 µg/ml FITC-transferrin in DMEM for 1 h. Excess transferrin was removed by washing; cells were scrapped off the dish, resuspended in PBS, and immediately analyzed by flow cytometry. From the two peaks seen, the one with higher fluorescence was determined and was taken to correspond to the higher density of transferrin receptors as a result of dynamin²⁴⁺⁺ expression. From these measurements, it was inferred that 70% of the induced cells (subpopulation with high FITC intensity) were positive for exogenous dynamin expression.

**Endosome Labeling for Flow Cytometry—**To investigate the effect of K⁻ depletion on endosomal pH, control or K⁻/-depleted HeLa cells were incubated in K⁻/-containing or K⁻/-free buffer, respectively, containing 4 mg/ml Cy5-dextran (70 kDa) and 1 mg/ml FITC-dextran (70 kDa) for 20 min at 37 °C. Cells were washed three times with ice-cold PBS and analyzed immediately by flow cytometry (32). To measure the endosomal pH in dynamin²⁴⁺⁺ and dynamin²⁴⁺⁺⁺ cells, they were incubated at 37 °C in DMEM containing 4 mg/ml FITC-dextran (70 kDa) and 1 mg/ml Cy5-dextran (70 kDa) either for 20 min or for 5 min, followed by incubation in dextran-free DMEM for 15, 55, and 115 min, respectively. Cells were washed three times with ice-cold PBS and analyzed immediately by flow cytometry.

**Generation of a pH-standard Curve for Flow Cytometry—**Standard buffers with pH values between 5.0 and 7.5 were obtained by mixing 50 mM HEPES with 50 mM MES, both containing 50 mM NaCl, 30 mM ammonium acetate, and 40 mM sodium azide. A pH calibration curve of internalized FITC/Cy5-dextran was generated using aliquots of the labeled cells (24). Cells were divided into eight aliquots, pelleted, resuspended in the various buffers, and equilibrated at room temperature prior to analysis. Under these conditions, cells are depleted of endogenous ATP; vacant ATPases do not function; and equilibration of intravesicular and external media is accomplished by weak acid-base buffers (24, 33).

**Flow Cytometry and Calculation of Endosomal pH—**A dual-laser FACSCalibur (Becton Dickinson Immunocytometry Systems) equipped with argon ion and red diode lasers was used. FITC fluorescence was measured using a 530-nm band-pass filter (30-nm bandwidth). Cy5 fluorescence (635 nm) was determined using a 661-nm band-pass filter (16-nm bandwidth). Each sample was measured eight times; samples in standard buffers were determined in duplicate. The mean values of FITC fluorescence and Cy5 fluorescence were calculated for each sample, and the autofluorescence of unlabeled samples was subtracted. The ratio of FITC to Cy5 was determined, and the mean pH of the labeled endocytic compartments was calculated using the pH-standard curve (24).
Internalization of FITC-Transferrin, 125I-Labeled β-VLDL, and FITC-Dextran into HeLa Cells Is Strongly Reduced upon Hypotonic Shock and K⁺ Depletion—Using the procedure leading to dissociation of clathrin coats from the plasma membrane of HEP-2 cells previously employed by Madshus et al. (18), internalization of HRV2 into HeLa cells was studied. Transferrin is generally accepted as a ligand, exclusively internalized via the clathrin-coated pit pathway (34, 35). Therefore, plasma membrane binding (at 4 °C for 1 h), as well as subsequent internalization (after warming to 37 °C), was determined with FITC-transferrin in control buffer and after K⁺ depletion. Although binding was slightly augmented (110% of the control value), internalization was inhibited to ~40% of the control value by K⁺ depletion (Fig. 1A).

Rabbit β-VLDL is preferentially internalized via LDLR if not enriched in apoE (36) and has also been shown to be internalized via clathrin-coated pits (10). Cell monolayers were incubated with 125I-labeled rabbit β-VLDL for 20 min at 37 °C to ensure localization in endosomes, but not in lysosomes (8, 14, 30); plasma membrane-bound ligand was removed; and internalized ligand was determined by γ-counting. As expected, hypotonic shock and K⁺ depletion reduced internalization into HeLa cells to ~30% of the control value (Fig. 1C).

Since we intended to use the fluid-phase marker FITC-dextran for intravesicular pH measurements, the influence of hypotonic shock/K⁺ depletion on its uptake was also investigated. Inhibition of clathrin-dependent endocytosis has been reported to either reduce or not affect fluid-phase endocytosis in different cell types (37, 52). Cells were incubated with FITC-dextran for 20 min at 37 °C, and cell-associated fluorescence was determined. As depicted in Fig. 1B, fluid-phase uptake into HeLa cells was reduced in K⁺-depleted cells to ~50% of the control, a value that compares well with that previously determined for rat fibroblasts (37).

Removal of Plasma Membrane-bound HRV2 Is Not Quantitative—Determination of HRV2 uptake requires removal of cell surface-attached virus. Therefore, the influence of K⁺ depletion on viral binding to the plasma membrane of HeLa cells was determined. Cells were incubated with [35S]methionine-labeled virus at 4 °C for 1 h, washed with PBS²⁺, and lysed; and cell-associated radioactivity was measured. HRV2 binding to K⁺-depleted cells was always found to be increased compared with control cells. However, it largely varied in five individual experiments using different viral preparations (between 120 and 300% of the control values). The increased binding might be caused by plasma membrane accumulation of the receptors resulting from impaired internalization or by an increase in receptor affinity as reported for binding of low density lipoprotein to LDLR (10). Next, the efficiency of various treatments to remove plasma membrane-bound virus was examined. Cells were incubated with 35S-labeled HRV2 as described above and washed with PBS²⁺, and total cell-associated virus was determined from an aliquot; this value was taken as 100%. The remainder of the cells were subjected to the procedures listed in Table I. After each treatment, the cells were washed with PBS²⁺, and cell-associated radioactivity was measured by scintillation counting. As EDTA specifically inhibits HRV2 attachment (38), nonspecific binding was determined in the presence of 10 mM EDTA. When present during incubation of the cells with virus, EDTA indeed strongly reduced surface binding (only 10 and 3% bound to control and K⁺-depleted cells, respectively); however, it failed to efficiently remove virus from the cells (with 59 and 38% of the virus remaining bound). The effect of washing with sodium acetate buffer adjusted to pH 5.0 was similar, and even trypsin at 2.5% was not able to quantitatively remove surface-bound virus.

At 4 °C, plasma membrane-bound virus should also remain accessible to antibodies. We thus tried to distinguish bound virus from internalized virus with the aid of mAb 8F5. This neutralizing antibody binds bivalently to a linear epitope on
the viral capsid protein VP2 and does not aggregate (29). Similarly to a polyclonal antiserum, it also recognizes 135 S and 80 S particles as well as denatured VP2; thus, it binds to all possible conformations of the virus. Cells with 35S-labeled HRV2 bound at 4 °C were incubated with mAb 8F5, and virus-antibody complexes were recovered by S. aureus aided immunoprecipitation, and immunoprecipitated virus was determined by liquid scintillation counting. Furthermore, generation of C-antigen is insensitive) fluid-phase markers for 20 min at 37 °C. After cooling, cells were washed and immediately analyzed by flow cytometry. The average pH of all endosomal compartments labeled under these conditions was then calculated from the ratio of the FITC and Cy5 fluorescence intensities based on a pH-standard curve (Fig. 3A). As shown in Fig. 3B, the average pH of dextran-labeled compartments was found to be 6.2 in control cells, whereas cells subjected to K+ depletion showed an average pH of 6.6. Thus, hypotonic shock/K+ depletion results not only in dissociation of clathrin coats from the plasma membrane, but also in a severe reduction of endosomal acidification by −0.4 pH units. Since these values represent an average of all compartments labeled under these particular conditions, at least some of them must acidify to pH ≤5.6, which is necessary for the conformational modification (39). The difference of 0.4 pH units might thus easily explain the reduction in C-antigenic virus seen under K+ -depleted conditions.

Effect of Inhibition of Clathrin-dependent Endocytosis on HRV2 Internalization—As washing with EDTA removed about half of the plasma membrane-bound virus from control and K+ -depleted cells and, at the same time, was less damaging to the cells than incubation with trypsin or low pH buffer (see Table I), this treatment was chosen for the study of HRV2 internalization. Control and K+ -depleted cells were incubated with 35S-labeled HRV2 for 20 min at 34 °C (the optimal growth temperature of human rhinoviruses) and washed with the respective buffers at 4 °C, and cell-associated virus was determined after EDTA treatment. Under these conditions, 3577 ± 293 cpm/well was associated with control cells, and 4590 ± 1371 cpm/well (mean ± S.D. of three experiments done in triplicate) was associated with K+ -depleted cells. Thus, 28% more virus was associated with K+ -depleted cells compared with control cells. As ~50% of the plasma membrane-bound virus can be removed by EDTA treatment (Table I), this difference presumably reflects only the elevated levels of HRV2 binding to the plasma membrane of K+ -depleted cells (see above). Thus, internalization appears to proceed almost undisturbed in K+ -depleted cells, suggesting clathrin-independent viral uptake.

Low pH-mediated Conversion to C-antigenic Particles Is Decreased in K+ -depleted Cells—After HRV2 internalization, structural modifications of the capsid are exclusively triggered by pH ≤5.6 within late endocytic compartments and can thus be correlated with viral internalization. As C-antigenic particles of HRV2 are also recycled into the supernatant, only 8% of the total virus was present in the form of C-antigenic particles, control cells. However, whereas under control conditions 74% of the total virus was C-antigenic under K+ -depleted conditions (Fig. 2). Since the conformational change reflects viral internalization into endosomes with pH ≤5.6, the substantial reduction in C-antigenic virus seen under K+ -depleted conditions suggests that internalized virus did not encounter a pH low enough to allow for structural modification.

The pH of Endocytic Compartments Is Increased upon K+ Depletion—To determine the endosomal pH, cells were incubated with FITC-dextran (pH-sensitive) and Cy5-dextran (pH-insensitive) fluid-phase markers for 20 min at 37 °C. After cooling, cells were washed and immediately analyzed by flow cytometry. The average pH of all endosomal compartments labeled under these conditions was then calculated from the ratio of the FITC and Cy5 fluorescence intensities based on a pH-standard curve (Fig. 3A). As shown in Fig. 3B, the average pH of dextran-labeled compartments was found to be 6.2 in control cells, whereas cells subjected to K+ depletion showed an average pH of 6.6. Thus, hypotonic shock/K+ depletion results not only in dissociation of clathrin coats from the plasma membrane, but also in a severe reduction of endosomal acidification by −0.4 pH units. Since these values represent an average of all compartments labeled under these particular conditions, at least some of them must acidify to pH ≤5.6, which is necessary for the conformational modification (39). The difference of 0.4 pH units might thus easily explain the reduction in C-antigenic particles recovered from K+ -depleted cells compared with control cells (13).

Is Productive Uncoating Affected by Hypotonic Shock and K+ Depletion?—Viral infection depends on release of the RNA from the capsid and its efficient transfer from endosomes into the cytoplasm (productive uncoating). Therefore, we asked whether K+ depletion has any effect on productive uncoating. Native virus is converted from 150 S to 135 S particles upon release of the innermost capsid protein VP4. Additional release of the RNA results in 80 S particles; both particles are C-antigenic and can thus not be distinguished by mAb 2G2 immunoprecipitation. Furthermore, generation of C-antigen is

### Table I

| Treatment/wash | PBS | Incubation with EDTA | PBS + EDTA | pH 5.0 buffer | Trypsin<sup>a</sup> | mAb 8F5<sup>b</sup> |
|----------------|-----|----------------------|------------|---------------|-------------------|---------------------|
| HRV2 remaining bound to control cells (%) | 100 ± 17<sup>c</sup> | 10 ± 5 | 59 ± 17 | 62 ± 18 | 45 ± 18 | 54 ± 15 |
| HRV2 remaining bound to K+ -depleted cells (%) | 100 ± 29 | 3 ± 1 | 38 ± 16 | 48 ± 5 | 13 ± 5 | 32 ± 10 |

<sup>a</sup> EDTA was present during incubation with HRV2.

<sup>b</sup> Cells were incubated with 2.5% trypsin for 60 min at 4 °C.

<sup>c</sup> Cells were incubated with mAb 8F5 for 1 h at 4 °C to attach to surface-exposed HRV2. After cell lysis, mAb 8F5 with bound virus was recovered by S. aureus aided immunoprecipitation, and immunoprecipitated virus was determined by liquid scintillation counting.

<sup>d</sup> HRV2 remaining cell-associated after washing with PBS<sup>2</sup> was set at 100%.
not necessarily correlated with productive uncoating; nevertheless, it is a sine qua non condition. Consequently, de novo synthesis of viral proteins might be taken as a measure for productive uncoating. However, hypotonic shock/K^-depletion interferes with cellular protein synthesis (40), which can be restored upon returning the cells to K^-containing medium (10). Therefore, we first determined the time required for protein synthesis to recover from the effects of K^-depletion. Following the procedure detailed under “Experimental Procedures,” protein synthesis in K^-depleted cells was found to be initially reduced to half of the control values, but recovered within 6 h. It then remained constant for up to 17 h (data not shown). Based on these results, the following protocol for the analysis of viral protein synthesis was established. Control and K^-depleted cells were challenged with HRV2 at a m.o.i. of 100 for 20 min at 34 °C in the respective buffers. To halt virus uncoating after return of the cells to normal medium (MEM), they were washed and resuspended in buffers containing 200 nM bafilomycin A1 to elevate the endosomal pH to neutrality (13). After incubation for 40 min, cells were transferred into methionine-free MEM containing 2% FCS and bafilomycin A1 to allow for regeneration of protein synthesis (see above). Six h later, [35S]methionine was added, and incubation was continued for a further 10 h (for experimental setup, see Fig. 4). Cells were washed and lysed, and viral proteins were immunoprecipitated with anti-HRV2 antiserum. Proteins were separated by SDS-polyacrylamide gel electrophoresis, and incorporation of radioactivity was quantified by densitometry of the bands corresponding to VP1, VP2, and VP3 as seen on the x-ray film.}

***Fig. 2.*** Conformational change in native HRV2 to C-antigenic subviral particles is decreased in K^-depleted HeLa cells. 35S-Labeled HRV2 was internalized for 20 min into control and K^-depleted cells grown in 12-well plates. Cells were washed with EDTA/PBS, and C-antigenic subviral particles were immunoprecipitated with mAb 2G2 in cell lysates and in the incubation medium. Native virus was then precipitated from the cell lysates with rabbit anti-HRV2 antiserum. Radioactivity in the precipitates was measured by liquid scintillation counting. Values indicated are the means ± S.E. from three experiments with eight parallel determinations are shown.

***Fig. 4.*** Viral protein synthesis is only slightly reduced upon infection of K^-depleted cells. Control or K^-depleted cells were challenged with HRV2 at a m.o.i. of 100 for 20 min at 34 °C. Cells were transferred to the respective bafilomycin A1-containing buffers and incubated for 40 min. Thereafter, buffers were replaced with methionine-free medium containing 2% FCS and bafilomycin A1. At 7 h post-infection, [35S]methionine was added, and de novo synthesized viral proteins were determined at 17 h post-infection by immunoprecipitation and SDS-polyacrylamide gel electrophoresis followed by fluorography. One representative fluorograph out of five individual experiments carried out in duplicate is shown. The experimental setup is indicated at the top.
result in a similar decrease in viral protein synthesis. However, the diminution of C-antigenic particles in K^+–depleted cells to 11% of the control value (Fig. 2) does not parallel the observed reduction in viral protein synthesis (80%). Thus, additional effects must account for this apparently augmented transfer of the genomic RNA into the cytoplasm in K^+–depleted cells.

Taken together, depletion of cellular K^+ not only leads to inhibition of clathrin-dependent endocytosis, but also differently affects HRV2 binding, endocytosis, structural modification, and productive uncoating. Therefore, viral production cannot be directly correlated with the extent of clathrin-dependent endocytosis after inhibition by hypotonic shock and K^+ depletion.

The pH of Endocytic Compartments Is Increased in HeLa Cells Overexpressing Dynamin^{K44A}—To avoid possible artifacts caused by the alteration of ion fluxes or membrane potential upon hypotonic shock and K^+ depletion, we analyzed HRV2 entry by an approach similar to that recently chosen by DeTulloe and Kirchhausen (20). These workers investigated the requirement of functional clathrin-dependent endocytosis for infection by several enveloped and non-enveloped viruses. HeLa cell lines expressing either exogenous dynamin^{wt} or dynamin^{K44A} both carrying a HA tag have been established previously (19). Although overexpression of dynamin^{wt} has no effect on internalization via coated pits and coated vesicles, coated pits fail to become constricted, and budding of clathrin-coated vesicles is inhibited upon induction of dynamin^{K44A} overexpression. As a consequence, clathrin-dependent endocytosis is blocked. It is known that exogenous dynamin is not homogeneously expressed within the cell population (41), and only those cells (70–80%) that overexpress dynamin^{K44A} are completely blocked in clathrin-dependent endocytosis. Using immunofluorescence microscopy, the cells that express the mutant dynamin could be observed individually for viral synthesis after infection at a low m.o.i. (<1) (20). As pointed out above, HRV2 uncoating requires exposure to a low pH environment for capsid modification and subsequent infection (14, 30). Therefore, it appeared important to first assess whether overexpression of dynamin^{K44A} has any influence on endosomal pH.

Endosomal compartments were labeled by continuous internalization of FITC- and Cy5-dextran into dynamin^{wt} and dynamin^{K44A} cells. Cells were cooled, washed, and immediately analyzed by flow cytometry. The pH values of all endosomal compartments labeled under these conditions were then calculated from the FITC/Cy5 ratio of the fluorescence intensities based on a pH-standard curve (see Fig. 3A). Although fluid-phase uptake of dextran was found to be reduced to ~70% in dynamin^{K44A} cells compared with dynamin^{wt} cells, the average pH of the labeled compartments was increased by 0.7 units in dynamin^{K44A} cells (Fig. 5A). To investigate whether endosomal and/or lysosomal acidification was affected, pulse-chase experiments were carried out. Endosomes were pulse-labeled with FITC- and Cy5-dextran by co-internalization for 5 min at 37 °C, followed by a chase in dextran-free medium for the times indicated in Fig. 5B. The average intravesicular pH gradually decreased from 6.5 at 5 min to ~5.0 at 120 min in both cell types. However, that of dynamin^{K44A} cells was significantly higher at 15 and 55 min of chase with respect to the values measured in dynamin^{wt} cells. This suggests that the pH in early endosomes and lysosomes is unaffected, whereas acidification of late endosomes is altered most severely. This finding precluded experiments involving the measurement of de novo viral synthesis since the elevated pH in late endosomes of mutant cells was expected to inhibit the conformational change required for uncoating. Consequently, internalization of HRV2 into dynamin^{wt} and dynamin^{K44A} cells was analyzed first.

**Fig. 5.** The endosomal pH is increased in HeLa cells overexpressing dynamin^{K44A}. Dynamin^{wt} (WT) and dynamin^{K44A} (K44A) cells were incubated with FITC-dextran and Cy5-dextran in DMEM for 20 min at 37 °C (A) or for 5 min at 37 °C (pulse), followed by a chase by further incubation in DMEM without dextran (B). Cells were cooled, washed with PBS, and analyzed by flow cytometry. Endosomal pH was calculated based on a pH calibration curve (for details, see “Experimental Procedures” and the legend to Fig. 3A). The means ± S.E. from three individual experiments, each comprising eight determinations, are shown.

**Internalization of HRV2 into Dynamin^{wt} and Dynamin^{K44A} Cells—Lack of transferrin internalization into cells overexpressing dynamin^{K44A} (but not into control cells) is shown in Fig. 6A. Whereas dynamin^{wt} cells internalized transferrin irrespective of overexpression of dynamin^{wt}, cells overexpressing dynamin^{K44A} were clearly negative for transferrin staining. Binding and uptake of HRV2 were then followed by indirect immunofluorescence using mAb 8F5 and Alexa 488-labeled anti-mouse antibody. Exogenous dynamin expression was monitored via rhodamine-labeled anti-HA antibody. First, binding of HRV2 to the cells was monitored. Incubation was for 1 h at 4 °C, whereupon the cells were washed, and cell surface-bound virus was visualized (Fig. 6B). Under these conditions, a typical plasma membrane staining (small punctate and homogenous distribution over the entire cell surface) was observed. Next, HRV2 internalization was investigated. Since we found that virus could not be efficiently removed from the cell surface (see above and Table I), HRV2 remaining at the plasma membrane was masked with rabbit antiserum prior to incubation with mAb 8F5. Control experiments confirmed that virus bound to the plasma membrane could be rendered inaccessible to mAb 8F5 using this procedure (data not shown). Dynamin^{wt} and dynamin^{K44A} cells were incubated with HRV2 for 20 min at 34 °C and cooled; plasma membrane-bound virus was masked; and cells were fixed, permeabilized, and processed for immunofluorescence. As shown in Fig. 6C, cells expressing dynamin^{wt} exhibited a HRV2 staining pattern characteristic for early and late endosomes that was clearly different from sur-
face binding (compare with Fig. 6B). In agreement with the experiments using K1 depletion, HRV2 was found to be internalized also into cells expressing dynaminK44A as verified by HA staining. At the level of resolution of fluorescence microscopy, internalization appeared to be slightly less in the cells overexpressing the mutant dynamin (arrows) with respect to cells not expressing the exogenous protein.

Further confirmation for intracellular localization of HRV2 was obtained by confocal fluorescence microscopy. An example of a peripheral and a central layer of 12-layer stacks through a HA-dynaminwt cell and a HA-dynaminK44A cell is shown in Fig. 7. In these images, HRV2 is clearly seen within peripheral and perinuclear endosomes regardless of whether dynaminwt or dynaminK44A was expressed. Nevertheless, upon examination of 145 individual dynamin K44A-expressing mutant cells, only 68% were found to internalize HRV2 (data not shown).

HRV2 Can Enter via LRP and/or VLDLR by a Clathrin-independent Pathway—HeLa cells express LDLR, VLDLR, and LRP, which all bind HRV2. Nevertheless, it is currently unknown which of these receptors is preferentially used for infection. RAP binds with high affinity to VLDLR (Kd = 0.7 nM) and LRP (Kd = 4 nM), but with much lower affinity to LDLR (Kd = 500 nM) (42) and has therefore been used extensively in competition studies. HRV2 binding and infection of familial hypercholesterolemia fibroblasts, which are devoid of LDLR, can be blocked by GST-RAP (6). Therefore, we determined whether GST-RAP is able to inhibit viral uptake in dynaminK44A-overexpressing cells. Dynaminwt and dynaminK44A cells were preincubated with GST-RAP at 4 °C for 60 min to block plasma membrane receptors. HRV2 was then added, and incubation was continued at 34 °C in the presence of GST-RAP for 20 min. Cells were washed and prepared for immunofluorescence. In several independent experiments, as exemplified in Fig. 6D, a marked reduction in HRV2 internalization by GST-RAP was seen regardless of dynaminwt or dynaminK44A overexpression. This specific effect of GST-RAP on HRV2 internalization strongly suggests that virus is internalized preferentially by LRP and/or VLDLR in HeLa cells expressing wild-type as well as mutant dynamin-1. The low numbers of LDLR molecules present as a result of growing the cells in 10% fetal calf serum containing enough cholesterol to down-regulate receptor expression thus do not appear to appreciably contribute to HRV2 internalization.

De Novo Viral Synthesis Occurs Regardless of Overexpression of Dynaminwt or DynaminK44A—To confirm viral internalization and uncoating by means of viral replication in dynaminK44A cells, the synthesis of viral protein was monitored by indirect immunofluorescence after infection at a low m.o.i. of 1. Cells induced to overexpress either dynaminwt or dynaminK44A were infected with HRV2 at a m.o.i. of 100, and incubation was continued for 20 min at 34 °C. Cells were cooled, washed, and processed for immunofluorescence. Cells expressing HA-dynamin are indicated by arrows.
body. As shown in Fig. 8, cells stained positive for viral proteins irrespective of overexpression of the mutant dynamin.

**DISCUSSION**

Exposure of cells to hypotonic medium followed by incubation in the absence of extracellular potassium results in dissociation of clathrin coats from the plasma membrane (43). As a consequence, internalization is impaired for the receptors for low density lipoprotein, epidermal growth factor, transferrin, and other membrane proteins carrying cytoplasmic amino acid sequences that interact with the clathrin adapter complex AP2 (10, 18, 44).

Minor group rhinoviruses use members of the LDLR family, all possessing internalization signals, for cell entry. The currently accepted assumption that these receptors are exclusively internalized by the clathrin-coated pit pathway clearly conflicts with the observed data, an increase in the cytopathic effect after HRV2 infection of potassium-depleted cells (18). To clarify this apparent contradiction, we investigated the effects of K\(^+\) depletion on individual steps in viral infection. Having previously characterized the infection pathways of HRV2 and HRV14 in HeLa cells, these cells were chosen for analysis. In these cells, potassium depletion inhibited uptake of transferrin and \(\beta\)-VLDL, a potent LDLR ligand (Fig. 1). Under the same conditions, we found that HRV2 binding was increased, whereas internalization appeared to be almost unimpaired. The former effect might reflect a retention of receptors on the cell surface, as shown for canine parvovirus (45), or an increased affinity, as reported previously for LDLR (46); the latter finding, however, indicates that the virus and, consequently, its receptor(s) are indeed capable of cell entry in a clathrin-independent manner. Possible candidates mediating viral internalization under these conditions are LRP and VLDLR, as LDLR is clearly clathrin-dependent (10) (see below). When LDLR expression is down-regulated by cholesterol, there is still internalization of HRV2, possibly by other members of the LDLR family (6).

RNA release from the virion is triggered by the low pH prevailing in endosomal carrier vesicles and in late endosomes (13). It is intimately linked with a structural change in the viral capsid that can be detected with mAb 2G2, specific for C-antigenic subviral particles (30). Comparison of potassium-depleted and control cells with respect to the low pH-induced generation of C-antigen revealed that this step was reduced to \(\sim 11\%\) in the treated cells compared with the control cells. This was found to be due to an increase in the endosomal pH by \(\sim 0.4\) units (Fig. 3). The observed pH increase after K\(^+\) depletion is at variance with the findings of Madshus et al. (47), who reported that hypotonic shock leads to a temporary vesicular alkalinization, with the acidic pH being restored after return to isotonic medium. However, these workers internalized FITC-dextran for 6 h preceding the hypotonic shock and consequently recorded preferentially the pH changes taking place in lysosomes.

It was further established that viral protein synthesis after infection proceeds at an only slightly reduced level after potassium depletion (Fig. 4). Therefore, one has to assume that transfer of the viral RNA into the cytoplasm occurs with higher efficiency in potassium-depleted cells and thereby compensates for the lower efficiency of structural modification (generation of C-antigen) due to elevated pH. How this is brought about is currently unknown, but might be related to changes in the

**FIG. 7. Confocal images of internalized HRV2.** Dynamin\(^{wt}\) and dynamin\(^{K44A}\) HeLa cells were infected with HRV2 and processed for confocal immunofluorescence microscopy as described in the legend to Fig. 6. Images of 12 layers through the cell were recorded. Layer 11 (closer to the cell surface) and layer 6 (cut through the cell body) are shown. Upper panels, HRV2; lower panels, expression of the HA-tagged dynamins (as described in the legend to Fig. 6).

**FIG. 8. De novo viral synthesis occurs after infection of dynamin\(^{K44A}\) cells.** Cells overexpressing dynamin\(^{wt}\) (upper panels) and dynamin\(^{K44A}\) (lower panels) were infected with HRV2 at a m.o.i. of 1. Seventeen h post-infection, cells were fixed, and HRV2 was stained with mAb 8F5 followed by Alexa 488-labeled anti-mouse antibody. HA-dynamin expression was detected with rhodamine-labeled anti-HA antibody. Cells were viewed in a fluorescence microscope.
endosomal membrane potential facilitating translocation of the RNA. Hypotonic shock/K⁺ depletion therefore not only dissociates clathrin coats from the plasma membrane, but has multiple additional effects that need to be considered when interpreting data from experiments involving this treatment (47).

To avoid side effects of K⁺ depletion, internalization experiments were also performed with HeLa cells overexpressing either dynamin²⁴⁴ or the nonfunctional mutant dynamin²⁴⁴A. With these cells, DeTulleo and Kirchhausen (20) demonstrated the requirement of clathrin function for infection with the major group virus HRV14. By double staining immunofluorescence microscopy, these workers demonstrated that cells expressing the mutant dynamin (as detected via a HA tag) also synthesized viral protein (as detected via a virus-specific antiserum). HRV14 uncoating and subsequent de novo synthesis of viral protein can occur at neutral pH (8, 48). Uncoating of HRV2, however, is strictly dependent on pH ≤ 5.6, as demonstrated by complete inhibition of viral replication by monensin (30) or bafilomycin A₁ (14). Our finding of an increase in the mean endocytic pH in potassium-depleted cells thus called for caution and led us to determine the endosomal pH in HeLa cells upon induction of dynamin²⁴⁴ overexpression. Indeed, a substantial increase in the pH was found in late endosomal compartments, but not in lysosomes (Fig. 5). The latter finding is in accordance with recent reports on lysosomal degradation of proteins being unaffected in dynamin²⁴⁴-overexpressing cells (49, 50). Recently, association of dynamin-2 with late endosomes has been reported; this results in defective trafficking from late endosomes to the trans-Golgi network (50). As the membrane composition affects the endosomal pH, it is likely that a defect in membrane transport indeed leads to alterations in endosomal pH. We thus chose not to use viral replication as an indirect measure for viral uptake, but rather to determine internalized virus directly by immunofluorescence microscopy.

In accordance with the results of K⁺ depletion, virus was found to be internalized into cells overexpressing dynamin²⁴⁴A, as shown by staining with anti-HA tag antibody (Figs. 6 and 7). To distinguish between LRP-dependent and -independent internalization of the urokinase-type plasminogen activator receptor as a function of polarized or unpolarized growth of Madin-Darby canine kidney cells, competition with RAP was used (51). Evidence for LRP/VLDLR being capable of clathrin-independent internalization of HRV2 was thus also substantiated by competition with GST-RAP. In the presence of GST-RAP, the virus did not enter the cells, clearly indicating that interaction with LRP or VLDLR was involved in the process (Fig. 6D). Finally, clathrin-independent internalization and uncoating were confirmed by demonstrating de novo viral protein synthesis in the mutant cells (Fig. 8). Comparison with Fig. 6 reveals that the pattern of viral fluorescence was different. Whereas internalized virus appeared as perinuclear punctate staining, viral protein originating from viral replication was distributed evenly over the cytoplasm.

Carpentier et al. (52) demonstrated a reduction in internalization of the specific LRP ligand, α₂-macroglobulin conjugated to gold particles, into 3T3-L1 cells upon potassium depletion. Similarly, Sandvig et al. (53) showed that uptake of Pseudomonas exotoxin, another ligand of LRP, is severely reduced after potassium depletion in L cells and baby hamster kidney cells. These findings strongly favor internalization of these ligands by a clathrin-dependent pathway. This was recently confirmed by experiments demonstrating that the deletion of internalization signals in the cytoplasmic tail of LRP results in impaired internalization (54). On the other hand, it was shown that the toxicity of Pseudomonas exotoxin is enhanced under potassium depletion (55). Similar to the situation with HRV2, multiple effects of K⁺ depletion complicate the interpretation of these results. However, when intoxication is increased in the latter case, Pseudomonas exotoxin must at least be able to be internalized under conditions of K⁺ depletion. Therefore, LRP and/or VLDLR appears to be capable of being taken up into the cell in the absence of functional clathrin coats to some extent. Our results show that this occurs with substantial efficiency when HRV2 is bound.

Besides clathrin-dependent endocytosis, there are alternative access ways to the cell. For example, caveolae have been recently implicated in the uptake of various viruses (56–58); but caveolin expression is very low in HeLa cells, and caveolae can be excluded as entry vehicles (51, 59). Viral entry must thus occur via non-coated vesicles by a mechanism that is largely obscure. Contrary to the belief that glycosylphosphatidylinositol-anchored membrane proteins are taken up in caveolae, clathrin- and caveolin-independent endocytosis of a glycosylphosphatidylinositol-linked diphtheria toxin receptor in HeLa cells was demonstrated (59). Apparently, a pathway involving non-coated vesicles can be utilized by glycosylphosphatidylinositol-anchored proteins as well as by otherwise clathrin-dependent receptors, at least by LRP and VLDLR when their normal mode of entry is abolished by K⁺ depletion or expression of dynamin²⁴⁴A. Although our preliminary results indicate that RAP can be taken up as well in a clathrin-independent manner, it might be considered that endocytosis of the multivalent virus could be triggered in some way by multiple attachment of the receptors around the virion, thereby inducing receptor clustering.

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