

Virus-mediated Release of Endosomal Content In Vitro: Different Behavior of Adenovirus and Rhinovirus Serotype 2

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Abstract. Endosomal penetration by nonenveloped viruses might be accomplished by either local breakdown of the endosomal membrane (e.g., adenovirus) or formation of a membrane-spanning pore by capsid proteins. Uncoating of the nonenveloped virus human rhinovirus serotype 2 (HRV2) has been shown to occur from late endosomes and to be entirely dependent on the acidic pH in this compartment (Prchla, E., E. Kuechler, D. Blaas, and R. Fuchs. 1994. *J. Virol.* 68: 3713–3723). To investigate further the mechanism of uncoating of HRV2, an in vitro assay was established to test viruses or virus-derived peptides for their capacity to release cointernalized biotin-dextran of different molecular mass (10 and 70 kD) from isolated endosomes. The suitability of the assay was demonstrated by

use of a fusogenic peptide derived from influenza virus hemagglutinin (GALA-INF3). Whereas adenovirus induced a low pH-dependent release of up to 46% of the internalized biotin-dextran and did not show any significant size selectivity (as expected for endosome disruption), HRV2 mediated release of 27% of the 10 kD dextran and only traces of the 70-kD dextran. Similarly, GALA-INF3-induced release of biotin-dextran was also size dependent. The potential role of the capsid protein VP1 in HRV2 uncoating in vivo was also substantiated in our in vitro system using an amphipathic, NH₂-terminal peptide of VP1. Taken together, these data favor the model of a specific pore-forming mechanism for HRV2 uncoating which is in contrast to the membrane-disrupting mechanism of adenovirus.

FOR nonenveloped viruses the mechanism of viral entry into a host cell is far from being understood. Although physiological host cell functions such as receptor-mediated endocytosis are being exploited to gain access to the interior of a cell, the penetration of the endosomal membrane barrier by the viral nucleic acid requires tricky solutions. Conformational changes in the viral capsid proteins, either induced by interaction with the receptor and/or the acidic endosomal environment, most probably lead to the exposure of hydrophobic domains able to interact with the endosomal membrane (for reviews see Marsh and Helenius, 1989; Marsh and Pelchen-Matthews, 1993). Access of the viral genome to the cytoplasm is achieved either by disruption of the endosome or through a “pore” formed by amphipathic viral proteins (Rueckert, 1991).

The double-stranded DNA containing adenovirus has been extensively characterized; this virus attaches to a still unknown membrane receptor via the fiber protein (Philip-

son et al., 1968) but is internalized through vitronectin-binding integrins by receptor-mediated endocytosis (FitzGerald et al., 1983; Defer et al., 1990; Wickham et al., 1993). Subsequently, penetration into the cytosol from the early endosome (Greber et al., 1993) is triggered by the acidic pH prevailing in this compartment (Pastan et al., 1986). In the particular case of adenovirus, the endosomal membrane is disrupted, releasing internalized virus into the cytoplasm. Analysis by EM showed these cytoplasmic viral particles to be morphologically intact; however, recent biochemical and immunochemical data revealed the gradual dismantling of the virus during entry into the cytoplasm (Greber et al., 1993). Moreover, viral penetration results in the release of cointernalized proteins or DNA into the cytoplasm (FitzGerald et al., 1983; Pastan et al., 1986; Defer et al., 1990; Yoshimura et al., 1993). Adenovirus has also been shown to increase the permeability of the plasma membrane for small molecules when the cells are placed under mildly acidic conditions (Seth et al., 1984, 1985, 1987), and it can cause lysis of liposomes (Blumenthal et al., 1986) and the release of choline from isolated plasma membrane vesicles at low pH (Seth, 1994). As an interesting side effect of its membrane-disrupting properties, large DNA complexes also gain access to the cytoplasm, opening the way for efficient gene transfer into eukaryotes (Curiel et al., 1991; Wagner et al., 1992a;

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1. *Abbreviations used in this paper.* HA, hemagglutinin; HRV, human rhinovirus; KSH, K-acetate, sucrose, and Hepes.

Yoshimura et al., 1993). Although substantial indirect evidence for the disruption of the endosome and for the release of its content have accumulated, these processes have not been investigated in isolated endosomes so far.

The mechanism of uncoating appears to be different for members of the picornavirus family containing positive stranded RNA genomes. Specific receptors have been identified for poliovirus and for the major and the minor group of human rhinoviruses (Greve et al., 1989; Mendelsohn et al., 1989; Staunton et al., 1989; Tomassini et al., 1989; Hofer et al., 1994). These are clearly required for the initial attachment to the plasma membrane; the further steps necessary for the release of the RNA, however, are seemingly different for poliovirus and for each of the two groups of rhinoviruses. For poliovirus it has been shown that hydrophobic domains at the NH₂ terminus of VP1 are exposed as a result of receptor binding (Frick and Hogle, 1990; Kaplan et al., 1990). Additionally, the myristoylated innermost capsid protein VP4 is extruded during the capsid modification. Both the NH₂ terminus of VP1 (Frick and Hogle, 1990; Kirkegaard, 1990) and the externalized VP4 (Moscufo et al., 1993) have been shown to play an important role in the delivery of the viral RNA to the cytosol possibly by mediating the virus-membrane interaction. It has been suggested that the amphipathic helix at the NH₂ terminus of VP1 inserts into the membrane, leading to destabilization and local breakdown of the membrane or to pore formation (Frick and Hogle, 1990). Access of several membrane-impermeable compounds to the cytoplasm was observed in the early phases of poliovirus infection. Since the same effects were seen with adenovirus, this was taken to indicate that endosomal destabilization (i.e., endosome disruption; see Fig. 5 in Almela et al., 1991) occurred by essentially the same mechanism in these virus systems (Fernandez-Puentes and Carrasco, 1980; Otero and Carrasco, 1987). In contrast, more recent results with poliovirus support RNA translocation occurring via a pore by demonstrating that the coentry of α -sarcin into the cytoplasm is dependent on a proton gradient between the endosomal lumen and the cytosol. However, the interpretation of these results is difficult since the site and mode of entry of α -sarcin has not been characterized so far (Turnay et al., 1993). Moreover, the localization of poliovirus in endosomes during entry has never been shown unequivocally (Kronenberg et al., 1992). Nevertheless, uncoating of poliovirus itself is clearly independent of a low pH environment (Gromeier and Wetz, 1990; Perez and Carrasco, 1993).

Recently, we have shown that minor receptor group human rhinoviruses (HRV) are bound to the cell and internalized via the low density lipoprotein receptor and the α_2 -macroglobulin receptor/low-density lipoprotein receptor-related protein (Hofer et al., 1994). We have further investigated the internalization pathway and mechanism of uncoating of HRV2, a prototype virus of this receptor group (Neubauer et al., 1987; Prchla et al., 1994). After low endosomal pH <5.6 mediated conformational change of its capsid proteins, uncoating was shown to take place from a late endosomal compartment. Furthermore, the presence of a large proportion of viral particles devoid of RNA in isolated endosomes suggested that the RNA is translocated through a pore across the endosomal mem-

brane into the cytoplasm rather than by disrupting the endosome. Pore formation was further supported by the observation of empty capsids of HRV2 being rapidly degraded in lysosomes. Native virions that have failed to uncoat in the appropriate compartment also terminate in lysosomes (Lonberg-Holm and Korant, 1972; Neubauer et al., 1987; Prchla et al., 1994). This is clearly not the case for adenovirus (Greber et al., 1993), where endosome disruption prevents further transport to lysosomes. Nevertheless, direct methods to determine whether picornaviruses would generate pores that size-restrict the release of endosome entrapped material have not been used so far.

Pore formation by a nonenveloped virus has as yet been demonstrated only for reoviruses by electrophysiological studies. These viruses are proteolytically activated in the endocytic pathway or in the intestinal lumen, resulting in infectious subviral particles that induce anion-selective multisized channels when added to planar artificial lipid bilayers. These channels are similar to fusion pores formed by the influenza virus hemagglutinin (HA), suggesting the existence of fundamental mechanistic similarities between the processes of penetration by some of the enveloped and nonenveloped viruses (Tosteson et al., 1993).

Influenza HA is present in the envelope of influenza virus as a homotrimer that mediates receptor binding and membrane fusion (for reviews, see White, 1992; Wiley and Skehel, 1987; Stegmann et al., 1989). When exposed to acidic pH, HA changes its conformation, leading to the extrusion of apolar parts of the polypeptide chains, which are responsible for the fusion between the viral envelope and the endosomal membrane (White and Wilson, 1987; Bullough et al., 1994). Fusion is initiated by formation of a fusion pore having characteristics of a large ion channel and requires the transmembrane portion of HA (Spruce et al., 1991; Kemble et al., 1994). Peptides corresponding to the NH₂-terminus of influenza virus HA have also been shown to induce fusion of cholesterol containing liposomes in a pH-dependent fashion (Wharton et al., 1988). Peptide mediated release of small molecules like calcein from phosphatidylcholine unilamellar liposomes as well as from erythrocytes was also observed; whether this is due to membrane disruption or whether it occurs during fusion has, however, not been investigated (Wagner et al., 1992b). Moreover, these processes have not been studied under physiological conditions, that is, from isolated endosomes.

To assess whether minor group rhinoviruses are uncoated by a mechanism fundamentally different from that of adenovirus, we established a cell-free system for monitoring the release of internalized macromolecules from isolated endosomes. In addition, this system should allow for investigation of the effect of virus-derived peptides on the endosomal membrane. Compared with liposomes or plasma membrane vesicles, our method has the advantage of using purified endosomes, thus maintaining (almost) physiologic conditions. Determination of virus-mediated release of cointernalized markers of different molecular mass from isolated endosomes allowed a clear distinction between endosome disruption and pore formation to be made. Our data favor an uncoating model in which rhinovirus RNA is passed through the endosomal membrane via a pore of limited size.

Materials and Methods

Chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless noted otherwise. Bafilomycin A1, kindly provided by Prof. K. H. Altendorf (University of Osnabrück, Germany), was dissolved in DMSO at 20 mM and stored at -20°C . The final concentration of DMSO in the assays was kept below 1%. Avidin was purchased from Canadian Lysozyme, Inc. (Vancouver, Canada). Mouse antiadenovirus mAb 805 was purchased from Chemicon International, Inc. (Temecula, CA), and affinity-purified peroxidase-conjugated horse anti-mouse IgG (SA-10-522) was purchased from Zeneca (Zeneca Bio Products, Cleveland, UK). For Western blot detection of HRV2 mAb 8F5 (Neubauer et al., 1987) was used. Rabbit antidextran antiserum (Richter and Kågedal, 1972) was a generous gift of Dr. D. Kraft (University of Vienna, Austria), and Dr. A. Berglund (Pharmacia, Uppsala, Sweden).

Peptide Synthesis

The fusogenic peptide GALA-INF3 (GLF EAI EGF IEN GWE GLA EAL AEA LEA LAA GGS C; Plank et al., 1994), the control peptide (FLG IAE AID IGN GWE GME FGG GC; Plank et al., 1994), and the peptide corresponding to the first 24 amino acids of the NH_2 terminus of VP1 of HRV2 with an additional cysteine (NPV ENY IDE VLN EVL VVP NIN SSN C; Zauner et al., 1995) were assembled on a synthesizer (model 431A; Applied Biosystems, Inc., Foster City, CA) by using *p*-alkoxybenzylalkohol resin (0.97 mmol/g; Bachem, Bubendorf, Switzerland) as solid support and 9-Flourenyl-methoxy-carbonyl (Fmoc)-protected amino acids (Bachem) essentially as described (Plank et al. 1994). Peptide purification was as in Plank et al. (1994) and Zauner et al. (1995). GALA-INF3 and the control peptide were used with the thiopyridine protection group attached to cysteines.

Cell Culture and Viral Propagation

HeLa cells (Wisconsin strain; kindly provided by R. Rueckert, University of Wisconsin, Madison, WI) were grown in monolayers in MEM-Eagle (GIBCO BRL, Gaithersburg MD) containing heat-inactivated 5% FCS and 5% calf serum at 37°C ; in suspension culture, Joklik's MEM (GIBCO) supplemented with 7% horse serum was used. If not stated otherwise, all experiments were carried out with cells from suspension culture. HRV2 was propagated, labeled with ^{35}S -methionine in HeLa cells, and purified as described (Neubauer et al., 1987). Concentration of sucrose gradient-purified HRV2 was calculated from optical density at A260 ($1 \text{ U} = 9.8 \times 10^{12}$ virus particles/ml; Korant et al., 1972).

Adenovirus serotype 5 mutant dl 312, a replication incompetent strain deleted in the E1a region (Jones and Shenk, 1979) was propagated in the E1a *trans*-complementing cell line 293 (Graham et al., 1977) essentially as described (Curiel et al., 1991). Purification was achieved by CsCl gradient centrifugation, and the amount of viral particles contained in the virus band (refractive index 1.365) was calculated based on protein determination with BSA as standard (Bradford, 1976; 1 mg protein is 3.4×10^{12} virus particles; Lemay et al., 1980).

Endosome Labeling

For experiments aimed to determine leakage from without, cells ($2\text{--}5 \times 10^7$) were loaded with biotin-dextran at 12.5 mg/ml final concentration (from a 25 mg/ml stock solution in PBS) in 2 ml infection medium (MEM-Eagle containing 2% FCS and 30 mM MgCl_2) for 30 min at 37°C .

For leakage from within, cells ($2\text{--}5 \times 10^7$) were preincubated for 30 min at 37°C (for peptides and adenovirus) or 34°C (for rhinovirus) in the presence of 200 nM bafilomycin A1 to inhibit the vacuolar proton ATPase, resulting in a neutral pH in the endosomes. Thereafter, cells were resuspended in fresh infection medium plus 200 nM bafilomycin A1, and the respective peptide at a final concentration of 75 $\mu\text{g}/\text{ml}$ was added together with 12.5 mg/ml biotin-dextran. When leakage was induced by ATP-dependent acidification, bafilomycin was replaced by 70 mM ammonium chloride (pH 7.4) in all media (lower concentrations of ammonium chloride were ineffective to completely inhibit the conformational change and virus infection).

Alternatively, the respective purified virus was added at a multiplicity of infection of 1,000 (= 1,000 infectious particles per cell) together with biotin-dextran (12.5 mg/ml) and 200 nM bafilomycin A1. Cells were incu-

bated in 2 ml Eppendorf vials by rotation in a water bath. The following internalization conditions were used: peptides, 30 min at 37°C ; adenovirus, 15 min at 37°C ; and HRV2, 20 min at 34°C (the optimal growth temperature for HRVs). Thereafter, cells were rapidly cooled to 4°C and washed twice with 50 ml of cold PBS containing 10 mM EDTA, pH 7.4, to remove surface-bound virus (Lonberg-Holm and Whiteley, 1976; Wickham et al., 1993). Cointernalization of fluid phase markers (70 kD FITC-dextran, HRP) with HRV2 had no influence on the accumulation of the virus in endosomes as analyzed by free flow electrophoresis (unpublished observations). In addition, biotin-dextran did not affect virus infection.

Preparation of Endosome-enriched Golgi Fractions

Cells were homogenized in 4 vol 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 (ball size: 6.36 mm; Balch and Rothman, 1985) and a postnuclear supernatant was prepared by centrifugation at 1,000 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 M and 0.25 M sucrose in TEA buffer (Marsh et al., 1987). After centrifugation in a rotor (model SW 40; Beckman Instruments, Palo Alto, CA) 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 diluted to a concentration of 0.25 M sucrose and pelleted by centrifugation in a SW 40 rotor for 80 min at 20,000 rpm (42,000 g). The material was resuspended in 1 ml 0.25 M sucrose, 20 mM Hepes/TEA, pH 7.4, together with protease inhibitors (leupeptin, pepstatin, and antipain from 1,000 \times stock solutions in DMSO) at final concentrations of 1 μM with the aid of a 2 ml Dounce homogenizer with a tight-fitting pestle. Biotin-dextran-labeled endosomes in this Golgi fraction were enriched 90-fold with respect to the original homogenate. 50- μl aliquots of the endosome suspension were rapidly frozen in liquid nitrogen and stored at -70°C .

For leakage assays with the VP1-derived peptide or with adenovirus as well as for ATP-dependent acidification, fresh endosomes were used immediately after resuspension. For the determination of leakage induced by ATP-dependent acidification (Fuchs et al., 1989) endosomes were resuspended in acidification buffer (150 mM KCl, 5 mM MgCl_2 , 20 mM Hepes-tetramethylammonium hydroxide, pH 7.4).

Endosome Leakage Assay

To test the capacity of viruses and viral peptides to induce discharge of internalized biotin-dextran from isolated endosomes, the high affinity of avidin for biotin was exploited (Smythe et al., 1992). To capture biotin-dextran being released from endosomes, avidin was present in the incubation mixture at 0.5 ng/ml. First, the total amount of biotin-dextran was determined using 2–10 μl of the endosome suspension in a final volume of 50 μl by addition of 1% Triton X-100 and 0.1% SDS (final concentration; see ELISA assay, below). Based on the total amount of biotin-dextran present in the endosome suspension (= full signal = latent + nonlatent marker), between 8 and 20 μl of the endosome suspension was used per assay in order to obtain signals in the linear range (see ELISA assay, below).

Three different experimental setups were used:

1. Release from without (Fig. 1 A). 8–20 μl endosomes loaded with biotin-dextran of 10 or 70 kD were mixed on ice with 1–5 μl virus or peptide in KSH buffer (100 mM K-acetate, 85 mM sucrose, 10 mM K-Hepes, 0.2% BSA, adjusted with potassium hydroxide (KOH) to either pH 5.5 or pH 7.4), with 40 μl avidin (from a 5 $\mu\text{g}/\text{ml}$ stock solution in KSH, pH 5.5 or 7.5), and the final volume was brought to 200 μl with KSH of the respective pH. The peptides were added at final concentrations of between 4.4 and 50 $\mu\text{g}/\text{ml}$, whereas the respective viruses were added at concentrations ranging from 10^7 to 4×10^9 particles/ml (~ 20 pM to 8 nM) as stated in the figure legends.

2. Release from within (Fig. 1 B). Endosomes colabeled with biotin-dextran and virus or peptide in the presence of bafilomycin A1 (see above) were mixed with KSH buffer as above in the presence of 1 μM nigericin.

3. Release from within, active acidification (Fig. 1 C). Endosomes colabeled with biotin-dextran and virus in the presence of ammonium chloride (see above) were mixed with 5 mM ATP in acidification buffer (150 mM KCl, 5 mM MgCl_2 , 20 mM Hepes-tetramethyl ammoniumchloride, pH 7.4). As a control, 1 μM nigericin was added to dissipate any ATP-driven generation of a pH gradient.

In all cases, the leakage assays were carried out in glass tubes in a final volume of 200 μl . The reaction mixture was kept on ice, the first aliquot

ADDITION FROM WITHOUT

INTERNALIZED VIRUS OR PEPTIDE

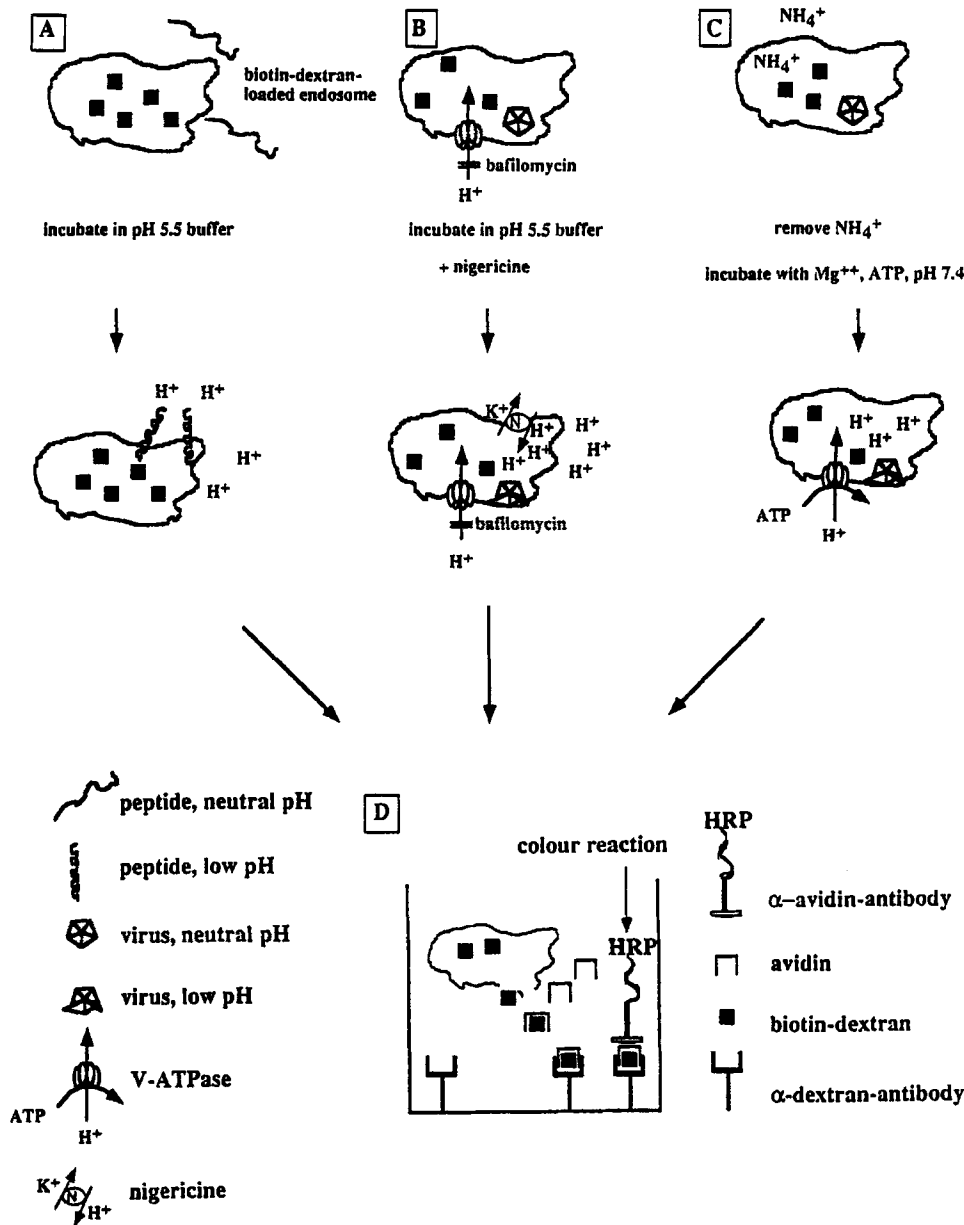


Figure 1. Experimental setup to investigate virus-mediated leakage of internalized biotin-dextran from endosomes isolated from HeLa cells. (A) Viruses or viral peptides were added to isolated biotin-dextran-loaded endosomes from without and acidification was by incubation in pH 5.5 buffer. (B) After dissipating the acidic pH in endosomes with baflomycin A1, viruses or viral peptides were cointernalized with biotin-dextran in the presence of this inhibitor of vacuolar proton ATPases. Isolated endosomes were then acidified by incubation with pH 5.5 buffer containing K-acetate and nigericin. (C) Viruses were cointernalized with biotin-dextran in the presence of ammonium chloride. After removal of ammonium chloride from isolated endosomes, the endosomal lumen was acidified via activation of the proton-ATPase by incubation in Mg-ATP containing buffer. (D) Released biotin-dextran was captured by avidin and the resulting complex was monitored by ELISA.

(50 μ l) was removed, and the reaction was started by transfer to a water bath of 37°C. Further aliquots were taken at 15 and 30 min. All samples were placed into vials (Micronic Systems, Lelystad, The Netherlands) containing 5 μ l stop solution (10% Triton X-100, 1% SDS, 0.5 mg/ml biocytin in KSH) and were vigorously mixed. Samples were kept at room temperature. 150 μ l blocking buffer (0.05 M NaCl, 0.01 M Tris/HCl pH 7.4, 0.001 M EDTA, 0.5% BSA, 0.1% SDS, and 1% Triton X-100) was added, and the samples were transferred into ELISA plates.

ELISA Assay. A capture ELISA was used essentially as described by Smythe et al. (1992) to quantitate avidin-biotin-dextran complexes. 96-well plates were coated with antidextran antiserum (Richter and Kågedal, 1972) diluted 1:2,000 vol/vol in 50 mM NaHCO₃, pH 9.6, for 1 h at 37°C or overnight at 4°C, blocked for 1 h at 37°C in blocking buffer, and could be stored in this buffer at 4°C for up to 1 wk. Plates were washed three times with PBS and incubated with the respective samples for 3 h at 37°C or overnight at room temperature. Thereafter, plates were washed in PBS followed by blocking buffer, incubated with 200 μ l per well HRP-conjugated antiavidin antibody (Dakopatts AB, Haegersten, Sweden; diluted 1:

1,000 vol/vol in blocking buffer) for 1 h at 37°C. HRP activity was determined with 200 μ l of *o*-phenylenediamine at 1 mg/ml and 0.4 μ l/ml 30% wt/vol H₂O₂ dissolved in assay buffer (3.65 g Na₂HPO₄, 2.82 g citric acid in 500 ml H₂O, pH 5.0). The reaction was halted by addition of 50 μ l of 2M H₂SO₄ and absorbances were determined using an ELISA reader (model MR 7000; Dynatech Laboratories Ltd, Sussex, UK) with 490 nm set as test wavelength and 630 nm set as reference wavelength. Absolute concentrations were determined with the aid of standard curves obtained with known concentrations of biotin-dextran on every ELISA plate. A₄₉₀ as a function of the concentration was linear up to 1.5 μ g/ml.

Results were expressed as percentage release of total (latent) internalized biotin-dextran in the respective endosome suspension at each time point according to the following formulas:

$$\text{release at pH 5.5} = \frac{x(5.5) - x_0(5.5)}{f_s(5.5) - x_0(5.5)} \times 100$$

$$\text{release at pH 7.4} = \frac{x(7.4) - x_0(7.4)}{f_s(7.4) - x_0(7.4)} \times 100$$

low pH dependent release =

$$\frac{x(5.5) - x_0(5.5)}{f_s(5.5) - x_0(5.5)} \times 100 - \frac{x(7.4) - x_0(7.4)}{f_s(7.4) - x_0(7.4)} \times 100$$

where

- x(5.5) is extinction at pH 5.5 at the respective time point
- x₀(5.5) is extinction at pH 5.5 at time = 0 (control at pH 5.5)
- x(7.4) is extinction at pH 7.4 at the respective time point
- x₀(7.4) is extinction at pH 7.4 at time = 0 (control at pH 7.4)
- f_s(5.5) is extinction at pH 5.5 after addition of detergents (full signal, pH 5.5)
- f_s(7.4) is extinction at pH 7.4 after addition of detergents (full signal, pH 7.4)
- f_s is full signal (release in the presence of detergent)
- f_s-x₀ is total (latent) internalized marker

Release due to nonspecific endosome breakage during incubation was always between 10 and 20% of latent internalized marker. Values were highly reproducible within a set of experiments but varied between different endosome preparations. Experiments were performed in quadruplicate and repeated two to three times with different endosome preparations.

Quantification of Virus in Isolated Endosomes by Western Blotting

100 or 20 μg protein of a representative endosome preparation labeled with adenovirus or rhinovirus, respectively, as used in the leakage assays, was precipitated by addition of 10 vol ice cold acetone, pelleted by centrifugation in an Eppendorf centrifuge (10 min at 12,000 g at 4°C), dried and dissolved in 15 μl Laemmli sample buffer, boiled, and separated on 12.5% SDS-polyacrylamide mini gels. The proteins were blotted onto nitrocellulose (0.2-μm pore size; Schleicher & Schuell Dassel, Germany), using a Nova-Blot Multiphor semidry blotting apparatus (Pharmacia) with 39 mM glycine, 48 mM Tris, 0.0375% (wt/vol) SDS, and 20% (vol/vol) methanol as electrode buffer at 0.8 A/cm². Transfer was for 90 min (HRV proteins) and for 180 min (adenovirus proteins). After blocking of the membrane in PBS, 0.5% Tween, 3% BSA (Blotto), proteins were detected by addition of the respective mAb (for HRV2, mAb 8F5; Neubauer et al., 1987; for adenovirus, mAb 805; Chemicon International, Inc.), and subsequent incubation with HRP-conjugated anti-mouse IgG (diluted 1:1,000 in Blotto) for 1 h. HRP was detected by use of the enhanced chemiluminescence HRP detection kit (Amersham Corp., Arlington Heights, IL) using the conditions as specified by the manufacturer. Bands of interest were quantified by laser densitometry of exposed x-ray films. Calibration curves were generated using known amounts of purified virus run in parallel on every gel. The stocks of purified virus contained 9 × 10¹¹ particles/ml of adenovirus and 3 × 10¹² particles/ml of HRV2, respectively.

Results

Peptide-mediated Release of Biotin-Dextran from Isolated Endosomes

Influenza virus HA2 NH₂-terminal fusogenic peptides have been successfully applied in receptor-mediated gene transfer protocols, leading to a substantial augmentation of the transfection efficiency (Wagner et al., 1992b). This has been attributed to a destabilizing effect on the endosomal membrane, allowing for facilitated access of the transfection complexes to the cytoplasm. Amphipathic peptides derived from viral envelope glycoproteins have been demonstrated to either promote fusion or disruption of liposomes at low pH depending on their composition and size (Wharton et al., 1988; Düzgünes and Shavnin, 1992). The

acidic amphipathic peptide GALA (Parente and Szoka, 1990), with its first 16 amino acids replaced by the NH₂-terminal sequence of influenza HA2 (= GALA-INF3) was among the most effective peptides in lysis of erythrocytes and liposomes in an acidic environment (Plank et al., 1994). Therefore, this peptide was used to establish an *in vitro* endosome leakage assay. Peptides can be applied from without due to their obvious independence from receptor binding and activation. Moreover, based on the results with liposomes (Wharton et al., 1988; Düzgünes and Shavnin, 1992) it can be expected that their action in fusion and/or disruption is independent on the asymmetry of the membrane bilayer. For these reasons, we attempted to demonstrate leakage of endosomal contents as a function of peptide concentration. To this end, endosomes were selectively labeled by internalization of the fluid-phase marker biotin-dextran, endosomes were prepared, and the appearance of extravesicular biotin-dextran as a function of the incubation conditions was taken as a measure of endosomal leakage.

Biotin-dextran was taken up by HeLa cells for 30 min at 37°C, conditions known to label all endosomal compartments. An endosome-enriched Golgi fraction was prepared (90-fold enriched in endocytic marker—biotin-dextran—with respect to the original homogenate), peptides were added at various concentrations, and the mixture was incubated in either low (5.5) or neutral pH (7.4) buffers (Fig. 1 A). Biotin-dextran released upon incubation of the isolated endosomes was captured with avidin, and the resulting biotin-avidin complex was quantitated by ELISA (Fig. 1 D). First, we determined the time and pH dependence of the release of 10-kD biotin-dextran by incubation with GALA-INF3 and with the control peptide (Plank et al., 1994). When GALA-INF3 was added at a concentration of 50 μg/ml (14.7 μM), release of up to 79% of total endosome-trapped (latent) internalized biotin-dextran could be detected at pH 5.5 after 30 min of incubation (Fig. 2 A), whereas at pH 7.4, much lower levels of dextran release were seen. In the presence of the control peptide, dextran release at pH 7.4 or at pH 5.5 even after 60 min was indistinguishable from background (incubation without peptide; see Materials and Methods). As demonstrated in Fig. 2 B, the low-pH-dependent release (release at pH 5.5 minus release at pH 7.4) of the dextran during a 30 min incubation period increased with increasing concentration of added GALA-INF3 up to 14.7 μM. This clearly extends the findings of the low-pH-dependent properties of the GALA-INF3 peptide from those originally observed in liposomes and erythrocytes (Plank et al., 1994) to isolated endosomes. Next, the pH-dependent discharge of biotin-dextran of 70 kD induced by GALA-INF3 was determined. Again, release was only observed at pH 5.5 (data not shown). However, the 10-kD biotin-dextran was released with threefold higher efficiency than the 70 kD dextran (Fig. 2 C). From these data one might infer that the interaction of the peptide with the membrane opens up pores of limited size rather than literally disrupting the endosome.

Having established the suitability of this assay for monitoring the release of internalized biotin-dextran from without, we then determined whether cointernalized GALA-INF3 was also able to promote the release from within. To

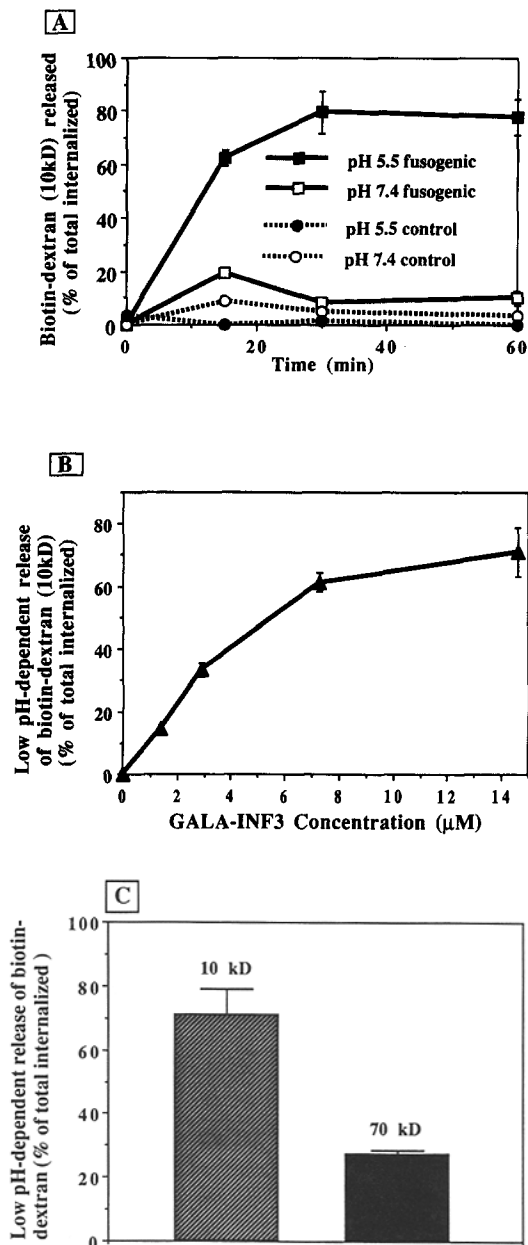


Figure 2. pH-, concentration-, and size-dependent release of biotin-dextran from isolated endosomes mediated by peptides from without. Endosomes were loaded with 10-kD biotin-dextran for 30 min at 37°C and isolated. The respective peptide was added to the endosomes in K-acetate buffer of pH 5.5 or 7.4 and incubated at 37°C. Release of biotin-dextran was quantitated by ELISA. 100% = total (latent) internalized biotin-dextran that was determined from the difference in the presence and absence of detergent. (A) Kinetics of peptide-mediated pH-dependent release of biotin-dextran. Peptides were present at 50 μg/ml. Data shown are the mean ± SD from three different experiments, each performed in triplicate. (B) Concentration dependence of biotin-dextran release mediated by GALA-INF3 during 30-min incubation (50 μg/ml GALA-INF3 = 14.7 μM). The values are expressed as low-pH-dependent release (release at pH 5.5 minus pH 7.4) in percentage of total internalized marker (see Materials and Methods). Data are from two independent experiments. (C) Size dependence of biotin-dextran release mediated by GALA-INF3. Isolated endosomes that were preloaded with biotin-dextran of 10 or 70 kD for 30 min at 37°C were incubated with 50 μg/ml GALA-INF3 in pH 5.5 or 7.4 buffer for 30 min at 37°C. Bi-

prevent any modification of the endosomal membrane during peptide internalization (Plank et al., 1994), the low endosomal pH has to be dissipated. Therefore, cells were preincubated with bafilomycin A1, a specific and irreversible inhibitor of vacuolar proton ATPases (Bowman et al., 1988), and the drug was also present during couptake of biotin-dextran and peptide. In addition, endosomes were isolated under conditions preventing active acidification. To activate the membrane-destabilizing effect of the internalized peptides, endosomes were then acidified by incubation in buffer of pH 5.5 in the presence of K⁺ and nigericin to allow for equilibration between the surrounding medium and the endosomal lumen (Fig. 1 B). Under these conditions, a release of up to 55% of total cointernalized dextran of 10 kD was observed when GALA-INF3 was present (Fig. 3). Release at pH 7.4 exerted by GALA-INF3 was only slightly higher than that of the control peptide regardless of the pH. GALA-INF3 is thus indeed capable of releasing endosomal content upon activation at low pH from within. Thus, release of cointernalized biotin-dextran can clearly be brought about by cointernalized peptides; however, the lower efficiency (25%; Fig. 3) compared with the release from without (70%; Fig. 2 B) might be due to the relatively low intraendosomal peptide concentration achievable via fluid phase endocytosis (see Discussion). Attempts to increase the amount of intraendosomal peptide by fluid phase uptake at peptide concentrations >22 μM in the medium failed, possibly because the peptide began to be toxic to the cells.

Endosome Rupture Induced by Adenovirus

Having demonstrated the applicability of this assay for the investigation of the effect of viral peptides on endosome integrity, we next determined the effect of adenovirus on endosomes. Adenoviruses of the subgroup C (AD2 and AD5) have been shown to disrupt the membrane of endocytic vesicles, thereby releasing cointernalized molecules such as proteins and nucleic acid into the cytoplasm (FitzGerald et al., 1983; Pastan et al., 1986; Defer et al., 1990; Curiel et al., 1991; Yoshimura et al., 1993). Disruption of the membrane is strongly favored by the low pH of the endosome (Seth et al., 1984; Greber et al., 1993). We therefore wanted to investigate whether the membrane disruption brought about by adenovirus *in vivo* could also be demonstrated in isolated endosomes. Thus, the interaction of adenovirus with the membrane of isolated endosomes was investigated by three different approaches: (a) Addition of the virus to endosomes loaded with biotin-dextran of 10 or 70 kD, respectively, from without (Fig. 1 A); (b) Cointernalization of the virus with the respective biotin-dextrans in the presence of bafilomycin A1 (Fig. 1 B) to inhibit *in vivo* endosome penetration. In both cases, release was initiated by acidification with pH 5.5 buffer. When adenovirus was trapped inside the endosomes, nigericin was added to allow for pH equilibration; and (c) Cointernalization of the virus with the respective biotin-

otin-dextran release was determined as in Fig. 1. Data are expressed as release (at pH 5.5 minus pH 7.4) in percentage of total (latent) internalized marker. Means ± SD from two independent experiments, each carried out in triplicate, are given.

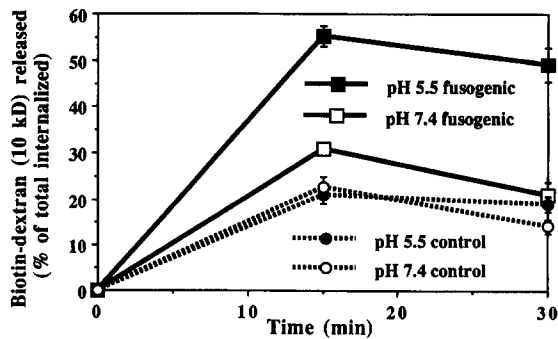


Figure 3. Release of biotin-dextran (10 kD) from isolated endosomes mediated by cointernalized peptides. Before internalization, the endosomal pH was dissipated with 200 nM bafilomycin A1. Peptides (75 $\mu\text{g/ml}$) were cointernalized with biotin-dextran of 10 kD in the presence of bafilomycin A1 for 30 min at 37°C. Release of biotin-dextran from isolated endosomes was initiated by incubation in K-acetate buffer (pH 5.5) containing 1 μM nigericin at 37°C; quantification was by ELISA, and data are expressed as in Fig. 2 A. Data are the means \pm SD of two independent experiments, each carried out in triplicate.

dextran in the presence of ammonium chloride. After removal of ammonium chloride from the isolated endosomes, acidification via the endosomal proton-ATPase was initiated by incubation with ATP.

When adenovirus was present in the incubation medium ($10^7\text{--}4 \times 10^9$ particles/ μg endosomal protein), release of 10-kD biotin-dextran was virtually background at pH 5.5 as well as at pH 7.4 (data not shown). This is most probably due to the lack of receptors on the outside of the vesicles or the asymmetry of the endosomal membrane. However, when the virus had been cointernalized into endosomes, up to 86% of total internalized 10 kD biotin-dextran was released at pH 5.5 after 30 min compared with 23% at pH 7.4 (Fig. 4 A). This clearly indicates a strong pH dependence of the adenovirus catalyzed release reaction.

To investigate whether the virus-induced membrane destabilization led to a size-dependent discharge of endosomal contents, low-pH-dependent release of biotin-dextran of 10 and of 70-kD were compared. As shown in Fig. 4 B, release of both internalized dextrans at pH 5.5 was indistinguishable, as would be predicted for membrane disruption. Thus, we could confirm earlier *in vivo* data showing endosome disruption during adenovirus infection (FitzGerald et al., 1983; Greber et al., 1993) using our *in vitro* system.

To mimic *in vivo* conditions, adenovirus-mediated release of 10-kD biotin-dextran was further investigated using ATP-dependent active endosome acidification (Fig. 1 C). In these experiments, the irreversible inhibitor bafilomycin A1 was replaced by the permeable weak base, NH_3 being also present throughout endosome preparation. In contrast to bafilomycin, NH_3 can be completely removed by dilution and subsequent pelleting of the endosomes. After equilibration of isolated endosomes in the appropriate buffer, acidification was initiated by addition of Mg^{2+} ATP. As shown in Fig. 5, ATP-dependent endosome acidification *in vitro* can induce leakage of internalized 10-kD biotin-dextran to $\sim 28\%$ when compared with only 12% obtained in presence of the K^+/H^+ -ionophore nigericin that prevents the establishment of any pH gradient.

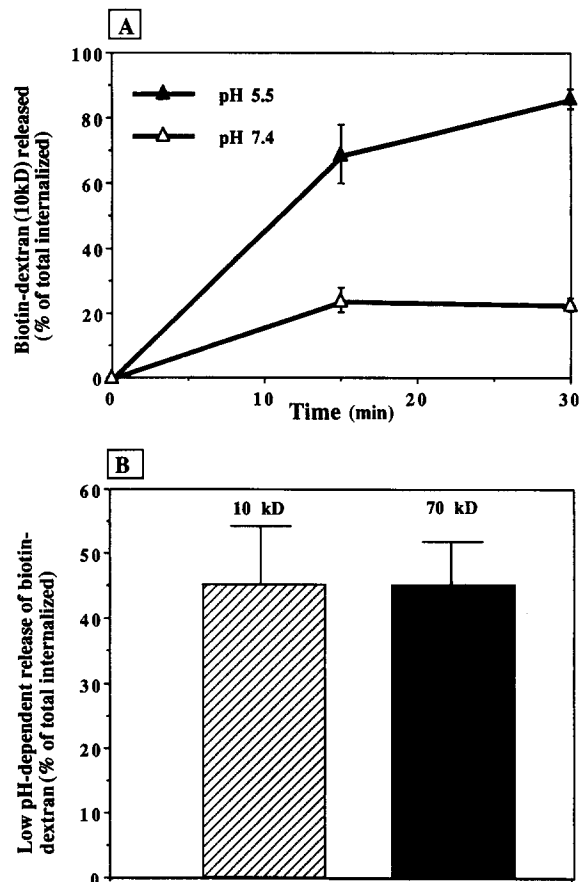


Figure 4. Adenovirus-mediated pH-dependent release of biotin-dextran from isolated endosomes from within: lack of size dependence. Adenovirus was cointernalized with 10-kD (A and B) or 70-kD biotin-dextran (B) in the presence of 200 nM bafilomycin A1 for 15 min at 37°C. Endosomes were prepared and incubated in buffer of neutral pH or were acidified by addition of K-acetate buffer (pH 5.5) and 1 μM nigericin. Data are the means \pm SD from three independent experiments and are given as (A) percentage of total (latent) internalized biotin-dextran or (B) low-pH-dependent release (see Fig. 2 B) after 15 min incubation at 37°C.

When the pH-dependent release during nigericin-mediated (passive) acidification (46%; see Fig. 4 A) and during active acidification (16%; see Fig. 5) is compared, it is evident that $\sim 34\%$ of the endosomes labeled with adenovirus can lower their pH below 6.0 *in vitro*, the pH threshold for membrane permeabilization (Seth et al., 1985).

HRV2-mediated Release of Biotin-Dextran from Isolated Endosomes

We have recently shown that HRV2 infection was clearly dependent on the low-pH environment prevailing in late endosomes; furthermore, appearance of 80S particles devoid of RNA in purified endosomes was prevented when acidification was inhibited. The presence of these RNA-less particles in isolated endosomes of infected cells has been taken to indicate that the RNA must have been extruded from the endosome via a specific pore (Prchla et al., 1994). To further substantiate this hypothesis, HRV2 and adenovirus were compared with respect to differential release of dextrans of 10 and 70 kD, respectively. First, it

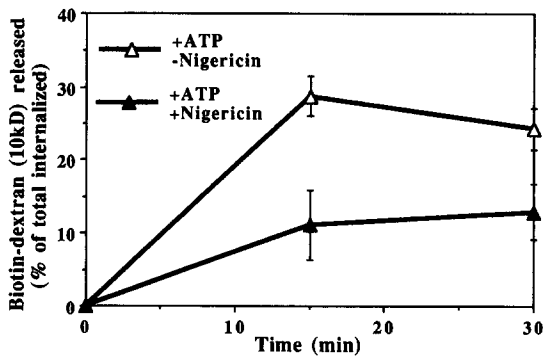


Figure 5. Adenovirus-mediated release of biotin-dextran (10 kD) induced by active endosome acidification (see Fig. 1 C). Adenovirus and biotin-dextran, 10 kD, were cointernalized into HeLa cells in the presence of 70 mM ammonium chloride at 37°C for 15 min. Endosomes were prepared and acidified after removal of the ammonium chloride by addition of Mg^{2+} ATP at 37°C. As a control, nigericin was added to dissipate the pH gradient between the endosomal lumen and the exterior. Released biotin-dextran was quantitated by ELISA and is plotted as the percentage of total (latent) internalized biotin-dextran. Data are the means \pm SD from three independent experiments.

was investigated whether HRV2 was able to release biotin-dextran of 10 kD from without in a pH-dependent manner. Similar to adenovirus, HRV2 failed to release internalized endosomal contents when added to an endosome suspension at neither pH (data not shown). Then, HRV2 and biotin-dextran (10 kD) were cointernalized under conditions preventing endosome acidification by bafilomycin A1 (see Fig. 1 B), and endosomes were prepared. As shown in Fig. 6 A, the release was found to be stimulated when the endosomes were incubated at low pH when compared with neutral medium. However, this low-pH-dependent stimulation of 10-kD biotin-dextran release was much less for HRV2 than for adenovirus (compare with Fig. 4 A).

Next, HRV2-induced release of biotin-dextran of 10 and 70 kD was compared. As shown in Fig. 6 B, the efficiency of release of 70-kD dextran was at least 10 times lower than that of 10-kD dextran. This is clearly different from the size-independent discharge caused by adenovirus (Fig. 4 B) and suggests that HRV2 fails to disrupt the membrane entirely but generates size-selective pores.

In analogy to the experiments undertaken with adenovirus, HRV2-mediated release of 10 kD biotin-dextran was further investigated using ATP-dependent active endosome acidification (Fig. 1 C). ATP-driven acidification by the endosomal proton pump was not sufficient to induce HRV2-mediated, low-pH-dependent release of biotin-dextran from isolated endosomes (data not shown). This might be attributed to the fact that only a very small percentage of the endosome population can lower their pH in vitro to <5.6 after removal of NH_4Cl (Prchla et al., 1994). This is the pH threshold for the conformational change that is a prerequisite for uncoating (Neubauer et al., 1987; Gruenberger et al., 1991). Isolated endosomes have been shown to give rise to only $\sim 10\%$ structurally modified HRV2 upon ATP-driven acidification compared with 85% under low-pH equilibrium conditions (pH 5.5 buffer; Prchla et al., 1994). Thus, about 11% of HRV2-labeled en-

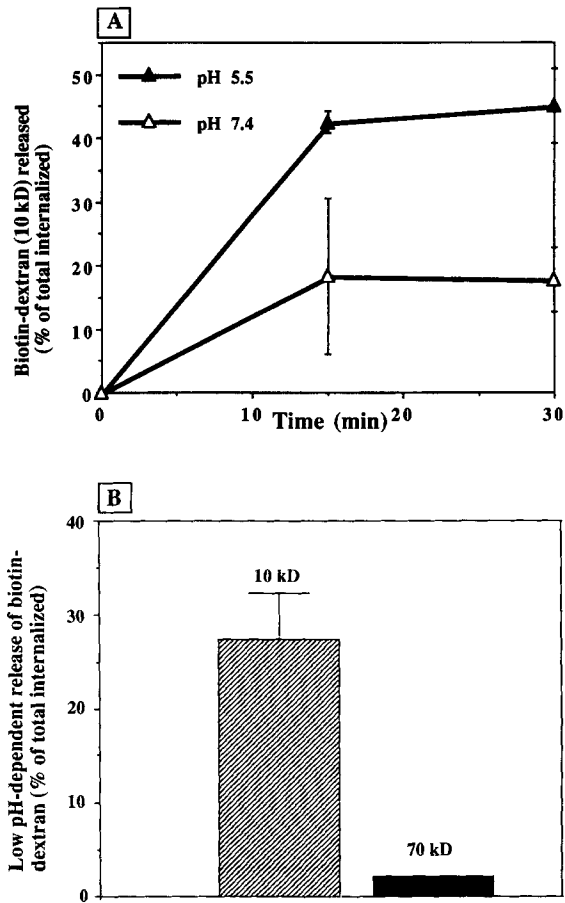


Figure 6. (A) HRV2-mediated pH-dependent release of biotin-dextran from isolated endosomes. HRV2 and biotin-dextran of 10 kD were cointernalized into HeLa cells in the presence of bafilomycin A1 at 34°C for 20 min. Endosomes were prepared and incubated at 34°C in buffer of neutral pH (control) or acidified by addition of pH 5.5 acetate buffer and nigericin. Released biotin-dextran was quantitated by ELISA and is plotted as the percentage of total (latent) internalized biotin dextran. Data are the means \pm SD of two independent experiments. (B) Size dependence of HRV2 mediated discharge of biotin-dextran from isolated endosomes. HRV2 was cointernalized with biotin-dextran of 10 or 70 kD into HeLa cells in the presence of bafilomycin A1 as in A. Release of biotin-dextran from isolated endosomes was measured after incubation at 34°C for 15 min in K-acetate buffer, pH 5.5, and 1 μ M nigericin. Release at pH 7.4 was subtracted (low-pH-dependent release; see Fig. 2 B). Data are the means \pm SD from two independent experiments, each performed in quadruplicate.

dosomes lower their internal pH below 5.6 when acidified via activation of the proton pump in vitro. This percentage is most likely too small to allow detection of biotin-dextran release above background levels. In addition, as NH_4Cl reduces transfer from early to late endosomes (see Discussion), the virus accumulates to some extent in early endosomes where acidification is limited (Fuchs et al., 1989).

Quantification of HRV2 and Adenovirus in Isolated Endosomes

Although internalization of the respective viruses was car-

ried out using the same multiplicity of infection of 1,000, comparison of the data obtained for adenovirus and HRV2 requires quantification of the amount of viral particles present in the respective endosome preparation. Therefore, endosomal protein was subjected to polyacrylamide gel electrophoresis in presence of SDS followed by Western blotting and quantification of viral protein by use of specific antibodies and the ECL system. Based on a standard curve made with known amounts of virus, the quantities of the respective viruses present in the endosomes could be estimated by laser densitometry of chemoluminographs. A typical preparation with a 90-fold enrichment of the internalized biotin-dextran in the endosome suspension with respect to the initial homogenate (see Materials and Methods) yielded an estimate of 7×10^6 adenovirus particles and 40×10^6 particles of HRV2 per microgram of total protein (Fig. 7). Thus, the different behavior of these two viruses cannot be attributed to gross differences in the number of viral particles being present at the same order of magnitude in isolated endosomes, but indeed reflects a virus specific mode of action.

The NH₂-terminal Peptide of VP1 of HRV2 Has Endosome-destabilizing Properties

Examination of the amino acid sequence of VP1 of HRV2 reveals a stretch of an amphipathic helix at the NH₂ termi-

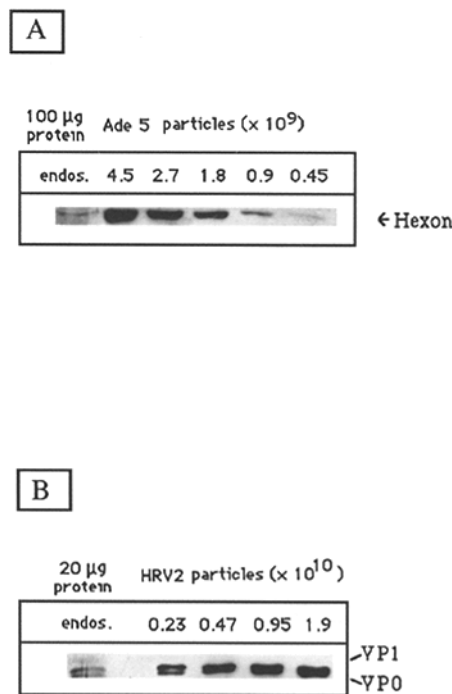


Figure 7. Quantitation of viral particles contained in isolated endosomes. Adenovirus (A) or HRV2 (B) at a multiplicity of infection of 1,000 were internalized into 5×10^7 HeLa cells for 15 and 20 min, respectively, and endosomes were isolated. Acetone-precipitated endosomal protein was separated by SDS-PAGE and blotted onto nitrocellulose. Viral proteins were quantitated as described in Materials and Methods. The correlation of the integrated peak area corresponding to the hexon protein (adenovirus) and VP1 (HRV2), respectively, with the number of particles was linear in the range shown ($R = 0.99$ for adenovirus as well as for HRV2).

nus. This protein segment is thought to be exposed upon interaction of the virus with membranes under low-pH conditions and has been shown for poliovirus to interact with liposomes (Fricks and Hogle, 1990). Therefore, a peptide corresponding to the first 24 amino acids of HRV2 VP1 was synthesized. Addition of this peptide to an endosome suspension loaded with 10-kD biotin-dextran from without (Fig. 1 A) resulted in a low-pH-dependent release (release at pH 5.5 minus release at pH 7.4) of the internalized marker (Fig. 8 A), whereas release of the 70 kD biotin-dextran was drastically reduced under the same conditions (Fig. 8 B). Thus, this peptide also exhibits size selectivity in the endosomal release reaction that is even more pronounced than that for GALA-INF3 (Fig. 2 C). Recent results on “transferrinfection” showed high efficiency of this particular VP1 peptide, whereas a peptide assembled with the same amino acid composition but scrambled sequence was ineffective (Zauner et al., 1995).

Discussion

We here present evidence for a different mechanism of uncoating of adenovirus and the minor receptor group human rhinovirus HRV2. An in vitro assay was established

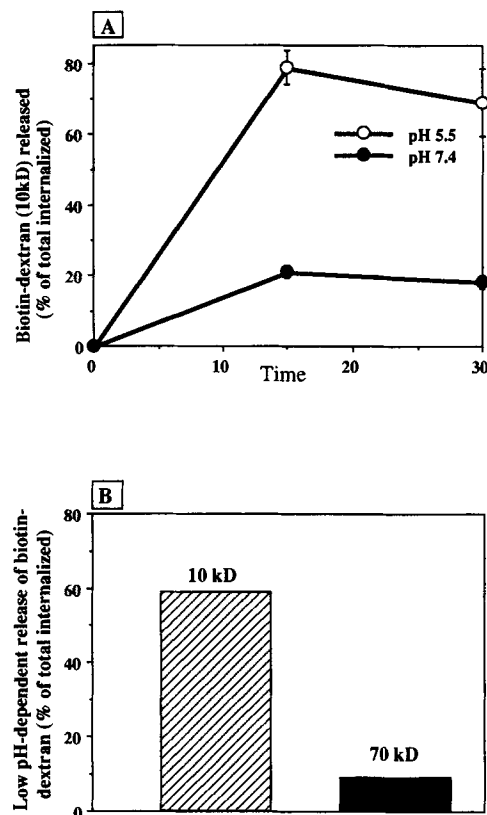


Figure 8. Effect of the VP1 NH₂-terminal peptide of HRV2 on release from without. Endosomes were labeled with 10-kD (A and B) or 70-kD (B) biotin-dextran for 30 min at 37°C. 43 µg/ml of VP1 peptide was added to the isolated endosome suspension in either pH 7.5 or pH 5.5 buffer and incubated at 37°C. Data shown are the means \pm SD from one experiment performed in quadruplicate and are given as (A) the percentage of total (latent) internalized biotin-dextran or (B) low-pH-dependent release after incubation for 15 min at 37°C.

that allows one to determine the release of an internalized fluid phase marker (biotin dextran) from isolated endosomes mediated by viruses and virus-derived peptides. Moreover, the use of dextrans of two different molecular masses enabled us to differentiate between endosome disruption and pore formation. A peptide known to induce leakage of liposomes and erythrocytes (GALA-INF3; Plank et al., 1994) indeed showed release of the internalized dextrans at low pH when added from without as well as when cointernalized (from within). Leakage of the 10-kD dextran was three times as much as that of the 70-kD dextran suggesting that this particular peptide is able to form pores of limiting size. As expected, both viruses investigated in this study were ineffective when added to the endosomes from without. However, when cointernalized with the fluid phase markers, adenovirus led to an almost size-independent release of endosomal contents. In contrast, HRV2-mediated biotin-dextran release is highly size dependent: 10-kD dextran was released to ~27%, whereas only 2% of the internalized 70 kD dextran was released in a low-pH-dependent manner.

Pore Formation by Amphipathic Peptides

Amphipathic peptides have been shown to permeabilize biological membranes as well as artificial liposome membranes. Depending on the lipid composition and the size of the liposomes used, the same peptides are also able to induce fusion of smaller vesicles (Düzgünes and Shavnin, 1992). Concomitant with the fusion event, leakage of small molecules has been observed under certain conditions (Wharton et al., 1988). We do not know whether GALA-INF3 induces fusion of the isolated endosomes when added from without. Therefore, at the present time we cannot differentiate between release during fusion or release independent of fusion. Nevertheless, a clear size dependence of peptide-induced release of biotin-dextrans was observed when the peptide was added from without. This is in accordance with previous data on the effect of the synthetic peptide GALA on small lipid vesicles: Parente et al. (1990) observed a pH-dependent leakage of fluorescent markers from lipid vesicles exhibiting clear-cut size dependent properties. Based on these experimental data, Parente et al. (1990) developed a mathematical model, in which a transbilayer channel with a diameter of 5–10 Å is formed by 8–12 GALA monomers. Whether the GALA-INF3 peptide used in this study forms similar channels or whether peptides with different composition and/or sequence exhibit a different behavior was beyond the scope of this study. Nevertheless, our data strongly suggest that at least in this particular case a pore of limited size is formed rather than the endosomal membrane being ruptured.

Comparison of the low-pH-dependent release (release at pH 5.5 minus release at pH 7.4) from without (70%; Fig. 2 *B*) with that from within (25%, Fig. 3) indicates that the latter is less efficient even at a higher initial peptide concentration in the infection medium (22 μ M from within) compared with that in the incubation buffer (14.7 μ M from without). However, the final peptide concentration achieved in the endosomes is unknown. Assuming that the uptake of the peptide occurs via fluid phase endocytosis and not adsorptive endocytosis, one can roughly calculate

the concentration of a fluid phase marker in endosomes based on stereological analysis of endocytic vesicles in BHK cells (Griffith et al., 1989); depending on the localization in early or late endosomes (and lysosomes), this estimation would result in peptide concentrations of between 44 and 7.7 μ M, respectively. Since small changes in peptide concentrations lead to rather substantial differences in dextran release from without, the lower release efficiency from within might be simply explained by a lower peptide concentration within endosomes compared with that in the infection medium. However, if a higher peptide concentration should prevail in endosomes, other effects such as asymmetry of the membrane bilayer cannot be excluded at the present time. Moreover, peptide-induced fusion, which is not expected to be triggered from within, might also explain the higher efficiency from without. In any case, the validity of the assay for monitoring selective release of endosomal contents could be demonstrated. Our *in vitro* system clearly provides a useful tool for the determination of the mechanism of peptide- or virus-mediated permeabilization of the endosomal membrane mimicking *in vivo* conditions.

Low pH-dependent Endosome Disruption by Adenovirus

Since the infectious entry pathway of adenovirus is well characterized and has been shown to involve rupture of early endosomes (Pastan et al., 1986; Greber et al., 1993), this viral system was used in our *in vitro* assay for comparison with the unknown mechanism of uncoating of minor receptor group rhinoviruses. When adenovirus was added to the isolated endosomes (from without), no release of internalized endosomal marker was observed, whereas the virus was highly effective when cointernalized. In both experimental setups, virus was present in similar concentrations (10⁷ particles/ μ g protein within endosomes, 10⁷–4 \times 10⁹ particles in the outside medium); therefore, it can be excluded that the lack of effect was due to an insufficient amount of virus present. During the preparation of this article, Wickham et al. (1993) demonstrated the role of interaction of adenovirus via the penton base protein with α v β 5 integrin in plasma membrane permeabilization. This effect was low-pH induced (pH \leq 6.0), but could not be brought about by purified penton base proteins, demonstrating the necessity of cofactors for membrane permeabilization. Thus, the inability of adenovirus to permeabilize endosomes from without in our *in vitro* system might be accounted for by the lack of α v β 5 integrins (with the proper orientation) on the cytoplasmic surface of the endosomes as well as by the lack of endosome- or virus-specific factors.

The size-independent release of the dextran markers cointernalized with adenovirus (in the presence of bafilomycin or ammonium chloride) was indeed as expected from *in vivo* experiments. Release was strongly stimulated by low pH; although neither a pH nor an ion gradient across the endosomal membrane was maintained under our *in vitro* conditions, the low pH dependence of endosome disruption is also in accordance with the data obtained *in vivo* (Pastan et al., 1986). From data obtained during ATP-dependent acidification of the endosomal lumen, it became clear that our system was capable of

matching *in vivo* conditions rather closely. The pH attained was sufficiently low (≤ 6.0) to break open 16% of the endosomes (see Fig. 5), whereas under pH equilibrium conditions, 46% release was seen (see Fig. 4 A).

Pore Formation by HRV2 and VP1 Peptide

Examination of HRV2 for its capability to permeabilize endosomes yielded a picture largely different from that seen for adenovirus: dextran of 10 kD was released to a substantially higher extent than dextran of 70 kD under low-pH conditions. This strongly favors the hypothesis that the viral RNA is extruded into the cytoplasm via a pore of defined size. However, it is not clear at the present time whether this transfer takes place in a vectorial manner (i.e., starting with the 5' or 3' end); both would require that the viral capsid still be attached to the membrane. Upon exposure to low pH, rhinoviruses undergo major structural alterations: their capsid proteins become hydrophobic, and they lose their innermost capsid protein VP4 and become permeable to RNases (Lonberg-Holm and Korant, 1972). It has been suggested that virus capsid proteins at the fivefold axis of symmetry are inserted into the membrane (Rueckert, 1991); the viral interior might then also become accessible for endosomal contents via the remaining vertices of the icosahedron and thus might allow efflux of medium-sized molecules into the cytosol (see the proposed model shown in Fig. 9). This is certainly not the case for enveloped viruses where a tight fusion event prevents loss of endosomal material. Therefore, release of genetic material (nucleocapsid) from enveloped viruses is clearly different from the release of cointernalized markers mediated by virus-derived fusion peptides. The potential role of the capsid protein VP1 for membrane insertion and hence pore formation is substantiated by our data on the VP1 derived peptide exhibiting an effect similar to entire viral particles. Moreover, Zauner et al. (1995) have shown that this same peptide is also capable of enhancing gene transfer into eukaryotic cells by allowing the access of foreign DNA into the cytoplasm. Similarly, membrane leakage is also induced by other peptides with the propensity toward formation of amphipathic helices (Plank et al., 1994).

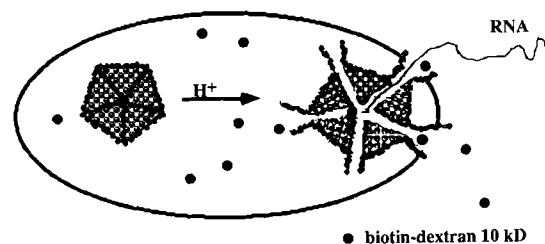


Figure 9. Proposed model for leakage of low-molecular-mass fluid phase markers (10-kD biotin-dextran) from endosomes mediated by membrane insertion and pore formation of HRV2 upon endosome acidification below pH 5.5. The model predicts that the viral capsid becomes permeable as a result of the conformational change and extrusion of VP4 and viral RNA. Openings are contiguous with the pores in the endosomal membrane. Material entrapped within the endosomes can thus find its way via the viral interior through the membrane pores into the cytoplasm.

Recently, reovirus was shown to give rise to anion-selective channels upon insertion into planar lipid bilayers (Tosteson et al., 1993). Fusion pores with similar conductance properties and diameter are also formed during the first seconds of fusion mediated by influenza virus HA with biological membranes (Spruce et al., 1991; White, 1992). The peptide GALA-INF3 used in our *in vitro* system gave rise to size selective pores and thus exhibited properties similar to HRV2. Consequently, it can be assumed that rhinoviruses implant into biomembranes very much like reovirus. Electrophysiological studies shall provide additional evidence for the formation of ion-selective channels by human rhinovirus.

Influence of Bafilomycin A1 on Transport of HRV2 through Endosomal Subcompartments

Clague et al., (1994) have recently shown that an active vacuolar proton pump is required for transport between early and late endosomes via carrier vesicles, since bafilomycin A1 blocked the formation of endosomal carrier vesicles in BHK cells. Nocodazole, which is known to inhibit fusion of carrier vesicles with late endosomes (Aniento et al., 1993) in BHK cells, is also able to inhibit uncoating of HRV2 (Prchla, E., D. Schober, D. Blaas, and R. Fuchs, unpublished observation) in HeLa cells. Since uncoating of HRV2 takes place in a late endosomal compartment (Prchla et al., 1994), one can assume that the vesicle shuttle model for endosomal transport is also operative in HeLa cells (Griffith and Gruenberg, 1991). Experiments to be published elsewhere have recently shown that bafilomycin A1 (200 nM) as well as ammonium chloride (70 mM) inhibit transfer of HRV2 and fluid phase markers (FITC-dextran and HRP) to late endosomes to the same extent. Part of HRV2 internalized in the presence of bafilomycin A1 is thus accumulated in early endosomes. It is therefore possible that at least part of the uncoating reaction as analyzed by our *in vitro* assay takes place from another subcompartment than uncoating *in vivo* (late endosomes). Nevertheless, adenovirus and HRV2 clearly differ in their mechanism of release of endosomal contents and therefore of the transport of their genomes into the cytoplasm.

Is Uncoating of HRV2 Driven by a pH Gradient between Endosomes and Cytosol?

Perez and Carrasco (1993) discussed the necessity of a proton motive force in order for some viruses to induce transport of the genetic material to the cytoplasm. Thus, in their view, the low pH by itself would not be sufficient in this respect. For HRV2, we did not see this requirement in our *in vitro* endosome leakage system, which does not, however, monitor viral RNA release directly. Moreover, Neubauer and colleagues (1987) have shown that infection of HeLa cells by HRV2 was totally prevented by monensin. However, when the cells were acidified by incubation with buffer at pH 5.5 after having been challenged with HRV2 in the presence of monensin, viral infection was restored. These results would thus suggest that uncoating does not require a pH gradient across the endosomal membrane. However, we cannot exclude that a pH gradient generated for a short time during the return of

the cells from pH 5.5 into neutral medium is sufficient to provide the driving force necessary for RNA translocation into the cytoplasm. We are currently extending our in vitro system to detect the viral RNA after the translocation process to determine the influence of pH gradient and pH equilibrium conditions on the productive uncoating of HRV2.

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