Site of Human Rhinovirus RNA Uncoating Revealed by Fluorescent In Situ Hybridization

Marianne Brabec-Zaruba,1 Beatrix Pfanzagl,1 Dieter Blaas,2 and Renate Fuchs1*

Department of Pathophysiology, Center for Physiology, Pathophysiology and Immunology, Medical University of Vienna, Waehringer Guertel 18-20, A-1090, Vienna, Austria, and Max F. Perutz Laboratories, University Departments at the Vienna Biocenter, Department of Medical Biochemistry, Medical University of Vienna, Dr. Bohr Gasse 9/3, A-1030 Vienna, Austria2

Received 5 February 2008/Accepted 9 January 2009

Human rhinoviruses (HRVs) are a major cause of the common cold. As Picornaviridae, they are nonenveloped with an icosahedral capsid assembled from 60 copies each of four proteins (VP1 through VP4) that encases a single-stranded positive-sense RNA genome (for a review of picornaviruses, see reference 28). The 99 currently characterized HRV serotypes are phylogenetically divided into two species; 73 are HRV-A, and 26 are HRV-B (17). Twelve serotypes of species A (the minor group) use members of the low-density lipoprotein receptor (LDLR) family for attachment. The remaining 87 types (the major group, including representatives of both species) infect via intercellular adhesion molecule 1 (ICAM-1) (31). As demonstrated for a few HRV types, the entry and uncoating pathways of minor and major group viruses differ considerably (1, 22).

As most of the data on minor group virus entry and uncoating have been obtained for HRV type 2 (HRV2) during its entry into HeLa cells. RNA uncoating of HRV2 is entirely dependent on low endosomal pH (≤5.6). When internalized into cells treated with bafilomycin, which results in neutralization of the endosomal pH, no FISH signal was recorded, whereas in the absence of the drug, fluorescent dots were seen. Therefore, FISH detects the genomic viral RNA only upon its release from the capsid. Free viral RNA was first seen at 10 min postinfection (p.i.) in the perinuclear area of the cell, which is indicative of RNA release in/from late endosomal compartments. Pulse-chase experiments and observation of HRV2 RNA and capsid proteins via microscopy, Western blotting, and reverse transcription-PCR revealed that the RNA signal persisted whereas the protein signal disappeared. This demonstrates transport of capsids to lysosomes and degradation. In contrast, viral RNA that had already been transferred into the cytoplasm escaped lysosomal breakdown as indicated by a persistent FISH signal. Taken together, our results demonstrate by direct means RNA arrival in the cytosol within 10 min p.i. Based on persistence of the FISH signal and productive infection in the presence of the microtubule-depolymerizing drug nocodazole, we localized this process to endosomal carrier vesicles/late endosomes.
first detected at between 10 and 12 min after virus internalization. Using various drugs that block distinct endocytic transport steps, we demonstrated that RNA release into the cytoplasm occurs from ECV in the perinuclear area. De novo-synthesized protein and RNA were seen at 3.5 h and 6.5 h p.i. as an endoplasmic reticulum-like pattern even when microtubules were depolymerized with nocodazole.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma (St. Louis, MO) unless specified otherwise. Bafilomycin A1 (Alexis Corp., Lausen, Switzerland), nocodazole, and wortmannin were dissolved in dimethyl sulfoxide at 20 mM, 20 mM, and 40 µM, respectively, and stored at −20°C.

Cells, buffers, and media. HeLa-H1 (HeLa) cells were grown in minimal essential tissue culture medium (MEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin G sodium salt, and 100 µg/ml streptomycin sulfate (all from Gibco Invitrogen Corp., Paisley, United Kingdom). HRV2, originally obtained from the ATCC, was propagated in HeLa cells, and the titers was determined as 50% tissue culture infectious dose (TCID₅₀) (3).

Internalization of HRV2. HeLa cells were grown on coverslips until half confluent and precultivated in serum-free MEM for 30 min at 34°C. Where indicated, 200 mM bafilomycin, 100 mM wortmannin, or 20 µM nocodazole (all final concentrations) was present. HRV2 at a multiplicity of infection (MOI) of 150 (i.e., 150 TCID₅₀/µl per well) was determined by comparison of both techniques) was internalized for 5, 10, and 12 min in fresh MEM (with or without the respective drug) at 34°C. Cells were washed twice with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde (PFA) in PBS at 4°C for 10 min, and prepared for indirect immunofluorescence microscopy or FISH. For pulse-chase experiments, virus was internalized for 12 min, nonattached virus was washed away with PBS containing 1 mM CaCl₂ and 1 mM MgCl₂, and the cells were further incubated in MEM. Prior to RNA isolation and Western blotting, they were briefly rinsed with PBS-1 mM EDTA.

Immunofluorescence microscopy. After fixation with PFA, cells were permeabilized with PBS containing 4% PFA and 0.2% Triton X-100 for 10 min. Samples were then quenched with 50 mM NH₄Cl in PBS. Blocking was performed with 10% goat serum in PBS (blocking buffer). HRV2 was detected with the monoclonal antibody 9F5 (10 µg/ml) directed against the viral capsid protein VP2 (30), followed by Alexa 488-labeled goat anti-mouse immunoglobulin G (IgG) (1:1000; Molecular Probes, Inc., Eugene, OR). First and second antibodies were diluted in blocking buffer; incubation was for 40 min at room temperature. Nuclei were stained with Hoechst dye 33342. Cells were mounted in a 9:1 (vol/vol) mixture of glycerol and 1 M Tris-HCl (pH 8.6) containing 25% diazobicyclo(2.2.2)octane (Merck, Darmstadt, Germany). Cells were viewed un-der a Zeiss Axioplan 2 fluorescence microscope using Axiovision software.

Quantification of degradation of viral RNA and protein. Images from the gel-resolved RT-PCR products and from the X-ray films exposed to chemiluminescence were analyzed by BioDoc-It software (UVP, Upland, CA). The results were expressed as percentages of control (untreated cells). Differences were considered significant at P < 0.05 using the Student’s t test for each condition compared to the respective control (untreated cells).

RESULTS

Endocytosis of HRV2 is very fast; after uptake via coated or uncoated vesicles, the virus arrives in early endosomes within the first 5 min (6). Shortly after, it reaches late acidic compartments where structural modifications of the viral capsid can be detected by using a conformation-specific monoclonal antibody. This transition from the native virion to empty capsids occurs at around 10 min p.i., as previously shown by immunoprecipitation from purified endosomes (23). We thus asked whether we could specifically detect RNA from the incoming virus as soon as it was released.

Free viral RNA is detected by the fluorescent RNA probe, but encapsidated RNA is not. Bafilomycin completely prevents the acid-induced conformational change of the viral capsid; consequently, RNA uncoating does not take place (2). Therefore, this drug was used to investigate whether our probe
hybridizes with encapsidated and/or uncoated viral RNA. HeLa cells were preincubated without (upper panels) or with (lower panels) 200 nM bafilomycin for 30 min, and HRV2 was internalized at 1,500 TCID<sub>50</sub>/cell for 12 min (with or without) at 34°C (pulse). Cells were washed and further incubated for the times shown (chase). After fixation, input RNA was detected with a riboprobe containing FITC-labeled UTP (left and middle panels), and viral capsid protein was visualized by indirect immunofluorescence using the monoclonal antibody 8F5 followed by Alexa Fluor 488-labeled goat anti-mouse IgG (right panels). Cells were viewed under a Zeiss Axioplan 2 fluorescence microscope. Bars, 10 μm.

FIG. 1. Uncoated HRV2 RNA is detected by FISH. HeLa cells were preincubated without (upper panels) or with (lower panels) 200 nM bafilomycin for 30 min, and HRV2 was internalized at 1,500 TCID<sub>50</sub>/cell for 12 min (with or without) at 34°C (pulse). Cells were washed and further incubated for the times shown (chase). After fixation, input RNA was detected with a riboprobe containing FITC-labeled UTP (left and middle panels), and viral capsid protein was visualized by indirect immunofluorescence using the monoclonal antibody 8F5 followed by Alexa Fluor 488-labeled goat anti-mouse IgG (right panels). Cells were viewed under a Zeiss Axioplan 2 fluorescence microscope. Bars, 10 μm.

However, as bafilomycin not only inhibits endosome acidification but also blocks the budding of ECV from early endosomes, the internalized virus remains in peripheral early compartments (2). The absence of RNA staining in the presence of bafilomycin demonstrates that RNA within the capsid is inaccessible to the probe; this makes it an excellent tool for the observation of the uncoated viral genome.

**Free viral RNA remains in the cytoplasm, but the viral capsid progresses to lysosomes.** We next asked whether the free RNA released from the incoming virus would be transferred to and remain in the cytosol. To this end, we took advantage of HRV2 transport to lysosomes; empty capsids and virus that has failed to uncoat arrive in lysosomes at about 25 min after uptake, where viral proteins and RNA are rapidly degraded (18, 23) (see Fig. 5 for a time course of HRV2 through endocytic compartments). Therefore, HRV2 was internalized for 12 min, and cells were further incubated in the absence of virus (chase) for 18, 48, and 108 min. A time-dependent decrease in stained viral proteins was seen following pulse internalization for 12 min. The fluorescence signal had virtually disappeared at a chase of 108 min (Fig. 2, upper panels). This indicates that the viral proteins had been almost completely degraded. Under these conditions, de novo viral protein synthesis is not yet detectable (see below). Cells infected by the same protocol were also stained for RNA. In contrast to the gradual decrease in fluorescence staining of viral proteins during the chase, the FISH signal persisted (Fig. 2, lower panels).

In addition, we also analyzed the degradation of viral protein and RNA by Western blotting and RT-PCR. Following the same protocol as described above, virus was internalized and chased for the times indicated in Fig. 3. Samples were subjected to SDS-polyacrylamide gel electrophoresis followed by detection of VP1 via a rabbit antiserum raised against the N-terminal 24 amino acid residues, a sequence that becomes exposed upon the conformational transition to subviral particles and is therefore particularly prone to digestion (16). Nevertheless, all viral proteins as well as the RNA are rapidly degraded.

![Image](http://jvi.asm.org/)

FIG. 2. Viral input RNA is still detected after lysosomal degradation of viral protein. HRV2 at 1,500 TCID<sub>50</sub>/cell was internalized for 12 min at 34°C. Cells were washed and further incubated in fresh medium for the times indicated (chase). Cells were fixed, and viral protein (upper panels) and uncoated RNA (lower panels) were then detected by immunofluorescence and FISH as detailed in the legend to Fig. 1. Note the strong decay of the signal of viral protein, which is due to lysosomal degradation, while the FISH signal is maintained. Bars, 10 μm.
degraded to trichloroacetic acid-soluble products once they arrive in lysosomes (13, 15, 18, 26). In a parallel experiment, total RNA was reverse transcribed, and the amount of viral plus-strand RNA relative to β-actin mRNA was determined by PCR. Figure 3 shows a representative Western blot of VP1 (Fig. 3A) and an ethidium bromide-stained agarose gel of the amplified cDNAs (Fig. 3B). The band of VP1 obtained immediately after internalization (12-min pulse and 0-min chase) quickly decreased up to 108 min (i.e., 120 min p.i.), when all VP1 had disappeared (Fig. 3A). The band then reappeared, and its intensity increased again with time because of de novo synthesis. Viral RNA also decreased but never disappeared, and the lowest level attained was roughly 30% of the input (12-min pulse and 108-min chase) (Fig. 3B). Here also an
increase is evident at later times, which is due to replication. As expected, lysosomal degradation was prevented by bafilomycin in both cases (Fig. 3A and B). The same experiments were also carried out with an MOI of 15 with essentially the same result. The individual experiments are summarized in the form of graphs in Fig. 3C to F. These results demonstrate that a large part of the RNA, as detected by FISH, has been released from the endosomes into the cytosol and did not arrive in lysosomes as did the capsid proteins. Furthermore, as the FISH signal remained roughly the same between 60 min (12-min pulse and 48-min chase) and 120 min (12-min pulse and 108-min chase) (Fig. 2), the viral RNA is apparently not degraded by cytoplasmic RNases (15, 26).

RNA release into the cytoplasm correlates with virus arrival in late endosomes. To pinpoint the site of RNA release, virus was internalized for 5, 10, and 12 min. An aliquot of the cells was fixed and stained for HRV2 proteins. As shown in Fig. 4A, viral capsid protein is clearly visible at 5 min p.i. At this time, virus is present mainly in peripheral vesicles and at the plasma membrane. At 10 and 12 min, viral protein is seen in endosomes close to the nucleus that had been identified as late endosomes by fractionation experiments and colocalization studies (2, 23). The other aliquot of the cells was processed for FISH; the results are depicted in the lower panels of Fig. 4A. The FISH signal and virus-specific antibody staining exhibit distinct kinetics of appearance: although internalized viral protein was evident at each time point, no RNA staining was seen at 5 min after internalization, a faint signal was seen at 10 min, and a strong signal appeared at 12 min. This is again evidence for the RNA being released in a time-dependent manner.

RNA release is delayed by wortmannin and unaffected by disruption of microtubules. As shown recently, the phosphatidylinositol 3-kinase inhibitor wortmannin does not affect virus internalization but leads to a delay in transport from early to late endosomes, in virus uncoating, in lysosomal degradation of the capsid proteins, and in HRV2 de novo synthesis (5). Therefore, we examined the effect of wortmannin on RNA uncoating via FISH by using a pulse-chase protocol similar to that used for Fig. 2. Indeed, in the presence of the drug, no RNA staining was seen at 12 min after internalization (compare Fig. 4B with A), but it was clearly visible at 60 min, when the virus still localized to peripheral enlarged endosomes (Fig. 4B, left panel). This is in line with RNA release being delayed by wortmannin, an effect also seen for productive uncoating and infection (5).

As shown previously, disruption of microtubules by nocodozole results in accumulation of the virus in ECV, intermediates between early and late endosomes that preferentially localize to the cell periphery. Conformational change and de novo viral protein synthesis were unaffected by the drug, as these compartments acidify to a pH similar to that of late endosomes. However, protein degradation is prevented, as ECV consequently do not deliver their cargo to lysosomes (2). Therefore, the influence of nocodazole on localization of the incoming RNA and the synthesis of new genomic RNA was studied. As depicted in Fig. 5A (upper panels), at 60 min p.i. viral protein was present in large, more peripheral vesicles, a typical staining pattern resulting from nocodazole treatment (2). Viral RNA preferentially accumulated at one side of the nucleus, whereas it was more disperse and more evenly distributed around the nucleus in the presence of the drug (lower panels). There are subtle differences in the staining patterns of RNA and viral capsids in the presence of nocodazole, supporting the above results on separation of the two viral constituents upon uncoating. At 222 min p.i. (i.e., 12-min pulse followed by 3.5-h chase) (Fig. 5B), viral de novo synthesis was apparent regardless of the presence of nocodazole. At 6.5 h, de novo-generated viral protein and viral RNA (Fig. 5C) gave rise to a staining pattern reminiscent of the endoplasmic reticulum (11; M. Brabec-Zaruba et al., unpublished data). This is in contrast to the case for poliovirus, where at 3.5 h p.i. newly synthesized non-structural protein 2B and plus-strand RNA were dispersed in the cytoplasm in nocodazole-treated cells. Nevertheless, neither production of virus progeny by poliovirus nor that by HRV2 is inhibited by nocodazole treatment (2, 11).

**DISCUSSION**

Using FISH for detection of plus-strand viral RNA and indirect immunofluorescence microscopy for detection of viral protein, we investigated the site of viral RNA uncoating. This was possible because the generated riboprobe did not hybridize with viral RNA as long as it remained within the intact capsid. This was inferred from the absence of the signal in the presence of bafilomycin, i.e., under conditions where the conformational change and RNA release do not occur. The RNA
became visible only after uncoating, giving rise to discrete fluorescent dots in the perinuclear area. Furthermore, after degradation of viral protein in lysosomes, the persistence of a strong FISH signal indicated that the RNA had been transferred into the cytosol. Based on the time-dependent progress of HRV2 through endocytic subcompartments (Fig. 6) and the use of drugs that block distinct transport steps as well as productive uncoating and infection (2, 5, 23), the appearance of the FISH signal correlated with virus arrival in late endosomes.

Although by fluorescence microscopy we cannot unequivocally demonstrate that the RNA is indeed in the cytoplasm (and not released into the lumen of the endosome) when the FISH signal appears, this is highly likely for the following reasons. (i) The signal remains in the same area of the cell starting from 10 to 12 min p.i. for up to 108 min; at this time the capsids have already progressed to lysosomes as seen from the disappearance of the protein signal (Fig. 2) due to degradation (23). (ii) The quantification of intact viral RNA and protein (Fig. 3) as a function of time (chase) after internalization (12-min pulse) shows complete disappearance of capsid proteins, while 30% of the input RNA remained intact at 120 min. (iii) The input viral RNA gives rise to bright, relatively large spots with an appearance similar to that of mRNAs encoding cytoplasmic proteins such as glyceraldehydes 3-phosphate dehydrogenase (21). It is of note that poliovirus RNA detected with FISH early after infection (0.5 to 1.0 h) exhibits similarly discrete spots in the cell periphery that later (1.5 h) give rise to large spots in the perinuclear area (10); as the Hogle group recently demonstrated RNA release into the cytoplasm from peripheral endosomes (7), these spots must be interpreted as stemming from input RNA. (iv) RNA replication that is known to take place on the cytoplasmic side of membranous structures (11, 25) results in an increased FISH signal for poliovirus at >2.5 h p.i (8, 10) as well as for HRV2 at >5.0 h p.i. (Fig. 5C) (Brabec-Zaruba et al., unpublished data). Finally, (v) release of nucleocapsids of Semliki Forest virus after low-pH-induced membrane fusion from endosomes into the cytoplasm gives rise to a grainy but not diffuse cytoplasmic staining pattern (29).

We have been using various reagents known to block distinct steps in the entry pathway in order to assess the correlation between productive uncoating (i.e., giving rise to infection) (2, 5) and RNA release as analyzed via determination of empty capsids. Employing the same drugs, we now analyzed these processes by using FISH. Bafilomycin, which completely blocks uncoating and infection, prevented the appearance of the FISH signal despite viral capsid proteins being detected in endosomes. Wortmannin, previously shown to delay virus transport from early to late endosomes and, as a consequence, viral replication (5), also led to a delay in RNA release. Finally, nocodazole, which depolymerizes microtubules and thus prevents transport of the virus from ECV to late endosomes, did not affect the intensity but affected the pattern of the FISH signal. This indicates that the RNA had been

![FIG. 5. Effect of nocodazole on RNA release and de novo synthesis of HRV2. Where indicated, HeLa cells were preincubated with 20 μM nocodazole for 30 min. Virus was then internalized for 12 min (with or without nocodazole) as for Fig. 2, followed by additional incubation with or without nocodazole for 48 min (A), 210 min (B), and 390 min (C), and cells were processed for separate detection of viral protein and RNA. In the absence of the drug, HRV2 proteins were present in perinuclear vesicles (A, top left panel) (arrow) compared to the large peripheral vesicles seen in the presence of nocodazole (A, top right panel) (arrow). RNA preferentially accumulated on one side of the nucleus (A, bottom left panel) (arrowhead), whereas it was more dispersed in the presence of the drug (A, bottom right panel) (arrowhead). Bars, 10 μm.](http://jvi.asm.org/ on September 23, 2017 by guest)
released from ECV and correlates with data showing that conformational change of the virion and productive infection occur in the presence of nocodazole (2). However, in contrast to the case for poliovirus, the location of newly synthesized HRV2 protein and RNA at 3.5 h p.i. was unaffected by disruption of microtubules (11).

In summary, we have successfully employed FISH to pinpoint the time p.i. and the site of release of the HRV2 genomic RNA into the cytosol and have correlated the present results with previous data on productive infection using different drugs. The noncovalently RNA-binding fluorescent dye Syto82 has been used to follow poliovirus RNA within the virus up to the point where it is being released from the capsid. Apparently, binding of this dye to poliovirus RNA is dependent on its secondary structure, as the fluorescent signal is lost after uncoating (7). In contrast, FISH, as used here, allows for detection of the HRV2 RNA as soon as it has left the virion. However, neither method can distinguish between the released RNA being still within the endosome (to be further transferred into the cytoplasm) or having already arrived in the cytosol. Notwithstanding, by showing by RT-PCR, Western blotting, FISH, and immunofluorescence microscopy that viral capsid proteins are entirely degraded whereas a substantial part of the viral RNA remains intact, we demonstrate by direct means RNA arrival in the cytosol within a time frame of 10 to 12 min and localize this process to ECV and late endosomes.

ACKNOWLEDGMENTS

We gratefully acknowledge the collaboration with Kurt Bienz and Denise Egger to establish the FISH procedure, and we thank Andrea Glaser for assistance in preparing the riboprobe. This work was supported by Austrian Science Foundation grant P-17590 to R.F.

REFERENCES


