The Rhinovirus Subviral A-Particle Exposes 3’-terminal Sequences of its Genomic RNA

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Enteroviruses, a large genus within the family Picornaviridae, undergo important conformational modifications during infection of the host cell. Once internalized by receptor-mediated endocytosis, receptor binding and/or the acidic endosomal environment triggers the native virion to expand and convert into the subviral (altered) A-particle. The A-particle is lacking the internal capsid protein VP4 and exposes N-terminal amphipathic sequences of VP1, allowing for its direct interaction with a lipid bilayer. The genomic ss(+)RNA then exits through a hole close to a 2-fold axis of icosahedral symmetry and passes through a pore in the endosomal membrane into the cytosol, leaving behind the empty shell. We demonstrate that in vitro acidification of a prototype of the minor receptor group of common cold viruses, human rhinovirus A2 (HRV-A2), additionally results in egress of the poly-(A) tail of the RNA from the A-particle, along with some 700 adjacent nucleotides. However, even after hours of incubation at pH 5.2, 5'-proximal sequences remain inside the capsid. By contrast, the entire RNA genome is released within minutes of exposure to the acidic endosomal environment in vivo. This finding suggests that the exposed 3'-poly-(A) tail facilitates the positioning of the RNA exit site onto the putative channel in the lipid bilayer, thereby preventing the egress of viral RNA into the endosomal lumen, where it may be degraded.

IMPORTANCE

For host cell infection, a virus transfers its genome from within the protective capsid into the cytosol; this requires modifications of the viral shell. In common cold viruses, exit of the RNA genome is prepared by the acidic environment in endosomes converting the native virion into the subviral A-particle. We demonstrate that acidification in vitro results in RNA exit starting from the 3'-terminal poly-(A). However, the process halts as soon as about 700 bases have left the viral shell. Conversely, inside the cell, RNA egress completes in about 2 min. This suggests the existence of cellular uncoating facilitators.

INTRODUCTION

Human rhinoviruses (HRV) are the major cause of usually mild but recurrent infections of the respiratory tract affecting everybody on the planet (1). The cause of the resulting discomfort is not cell lysis, but rather inflammation paired with a massive
production of mucus triggered by the induction of cytokines and reactive oxygen species. Although rarely serious, the disease results in decreased labor productiveness and lost working hours (2); therefore, HRV are of enormous economic importance (3).

In weaning infants and the elderly, infection can become life-threatening, particularly when affecting the lungs and in combination with asthma, chronic obstructive pulmonary disease, or cystic fibrosis (4). To date, efforts towards development of effective and well-tolerated drugs have not been successful. The currently available compounds have side effects that are only acceptable when the condition of the patient necessitates last-resort intervention (5, 6). Vaccination has been considered impracticable because of the existence of more than 160 different genotypes with poor immunological cross-reactivity (7); nevertheless, more recent research has identified antigenic epitopes shared among a number of HRVs that might be a starting point for the production of immunogens suitable for vaccination (8-10).

HRVs include three species, HRV-A, -B, and -C (11). They are phylogenetically closely related to the four human enterovirus species, which comprise more serious pathogens such as poliovirus and enterovirus 71 (EV71) among many others, all belonging to the genus Enteroviruses, family Picornaviridae, of ss(+)-RNA viruses (12). They possess an icosahedral protein shell of about 30 nm in diameter that is composed of 60 copies each of the capsid proteins VP1 through VP4. The genome is about 7200 nucleotides in length. At the 5'-end, instead of a cap structure, the genomic RNA carries a 21 amino acid long peptide referred to as VPg. A genome-encoded poly-(A) tract (13) of between 20 and 150 nucleotides in length (14) is present at its 3'-end.

In addition to the classification above, HRV-A and -B are divided into a minor and a major receptor group; the former group includes 12 HRV-A types which bind low-density lipoprotein receptors (LDLR) and related membrane proteins, whereas the latter group binds intercellular adhesion molecule 1 (ICAM-1) and comprises more than 89 representatives of HRV-A and HRV-B (15). A particular feature of the icosahedral protein shell is the canyon, a deep cleft encircling the star-shaped dome at the 5-fold axis of symmetry. Whereas the minor group receptors attach to the dome of the protein shell, ICAM-1 binds inside the canyon. The HRV-C receptor(s) is unknown.

Upon binding its respective receptor, the virus is taken up into the cell where it undergoes major structural modifications. Protein domains, in particular those of VP1 and
VP2, become displaced, resulting in expansion of the capsid by about 4% (16). Antiparallel helices of two adjacent copies of VP2 (residues 90 to 98) at the two-fold axes veer away from each other, resulting in the opening of pores. These movements are possible because of a hydrophobic pocket below the canyon of the protein shell; in native virus this void is occupied by a fatty acid that is released during uncoating, thus providing the necessary space for amino acid residues to move in (16). Cryo-electron microscopy 3D-image reconstructions (cryo-EM 3DR) of the related poliovirus during uncoating triggered by heating to 56°C demonstrated that the genomic RNA most probably exits from one of these pores (17). The size of the pores is sufficient only for passage of the nucleic acid as a single strand, suggesting that the genome must unwind prior to exiting the particle. This view is further supported by the finding that an intercalating dye pre-bound to the viral genome is stripped off during in vivo uncoating (18).

The conversion of poliovirus (19) and HRV-A2 (20) into A-particles results in additional density appearing close to the tips of the 3-bladed propeller at the 3-fold axes. This density is thought to stem from about 30 N-terminal residues of VP1; indeed, Fab fragments raised against a peptide derived from this segment were found to attach to this site (21). The amphipathic VP1 segment exposed in the A-particle might ’crawl up’ the canyon wall along the shoulder to the star-shaped dome; in the presence of cellular membranes, it presumably interacts with the lipid bilayer (22, 23). The exit sites of the N terminal VP1 sequences identified in poliovirus were shown to correspond to small channels in the expanded empty capsids of EV71 (24, 25) and HRV-A2. This observation suggests that the externalized segments retract upon completion of RNA exit (16).

By triggering HRV-A2 uncoating by heating to 56°C, as previously used in poliovirus studies on RNA release (17, 26), we recently demonstrated that RNA trapped inside the capsid takes on the shape of a thick rod pointing in the direction of a 2-fold and a 3-fold axis, at roughly opposite sides of the shell, suggesting a role in the “tail-first” (i.e. 3’-end first) release process (27). Particles with these internal structures were much more frequently observed when double-stranded regions in the RNA had been cross-linked with psoralen. This suggests that the RNA adopts this peculiar conformation when uncoating is halted and regions behind the exiting segment cannot be unwound. In our cryo-EM images of in vitro acidified HRV-A2, such density was not observed. This disparity implies that the
“condensation” of the RNA might be related to the (unphysiological) crosslinking and/or heating. Therefore, the role of this structure, if any, in in vivo uncoating is still enigmatic.

To assess whether RNA egress occurs with the same directionality under a setting more closely resembling the situation in the living cell, we repeated and extended our previous experiments using acidification, as naturally occurs in endosomes in vivo, instead of incubation at 56°C, to trigger the conversion of native virus into subviral particles.

MATERIALS AND METHODS

Chemicals, oligonucleotides, and enzymes were from Sigma unless otherwise noted.

Cells and virus. HeLa-Ohio cells were obtained from Flow Laboratories; they are now available from ECACC (Salisbury, United Kingdom). HRV-A2 was obtained from ATCC (VR482) and maintained in the laboratory. The following primers were used: “5’-end – reverse”: 5’-

dAAGGGTAAAGGTAGCCACATTCA-3’; “5’-end – forward”: 5’-

dGACCAATAGCAGTAATCAG-3’, “primer1 – reverse”: 5’-dAGCTTTACTTCCCTGCGCTG-3’;

“primer1 – forward”: 5’-dACCCTCTCAACCAGATACACT-3’; “M-oligo – reverse”: 5’-

daAGGTGTCAGTTATTTATGTAAGAGCTG-3’, “M-oligo – forward”: 5’-

dGCCCATGTGTGCAGTTTCC-3’; “primer2 – reverse”: 5’-dAATTGCTGTGTTAATTCC-3’;

“primer2 – forward”: 5’-dATGATGGTTATGAAAAAG-3’;

“primer3 – reverse”: 5’-dTCTTTGGTTATATAATTTTATAAGCTG-3’, “primer3 – forward”: 5’-

dTGTGGATAATTCAAACAAC-3’; “primer4 – reverse”: 5’-dAATTAATTCCCTTTCCAAT-3’

“primer4 – forward”: 5’-dGCAAAATCACTCCCTTCAAC-3’; “primer5 – reverse”: 5’-

daACACTAGCTGCGTTAAAACA-3’; “primer5 – forward”: 5’-dACGGCTAGAATGCTAATAC-3’; “3’-end 3 - reverse”: 5’-dCCACTATGCAAAAGCAAAT-3’, “3’-end 3 - forward”: 5’-

dCCCTCCCTAGAAATAATTAAAAT-3’; “3’-end 5 - reverse”: 5’-

dCCCTCCCTAGAAATAATTAAAAT-3’; “3’-end 5 - forward”: 5’-

dGAGGTGCTTTCTTATGTAATTCC-3’, “primer6 – reverse”: 5’-

Fluorescence Correlation Spectroscopy. FCS was carried out in a Confocal 1 instrument (Zeiss) as in previous work (27). The fluorescence autocorrelation function was determined for DyLight488-labeled oligonucleotides complementary to HRV-A2 genome sequences at the 3’-end (25-mer oligo-dT) and the 5’-end (position 443-468) in 75 mM KCl, 3 mM MgCl2,
50 mM Tris-HCl (pH 8.3) after the addition of native virus (control) and acidified and reneutralized virus, respectively. Between 10 and 50 independent measurements were taken, with a data acquisition time of 10 s each. Optimal molar ratios between oligonucleotide and free RNA (and the subviral particle with partially-exposed RNA) were as determined previously (27). HRV-A2 (at 1 µM) was mixed with the respective labeled oligonucleotide (at 50 nM). One vol of this mixture was acidified by adding 1.5 vol of 50 mM NaOAc (pH 5.2), incubated at 34°C, and samples (10 µl) were withdrawn at the times indicated in the Figures and reneutralized on ice via addition of 10 µl 100 mM Tris-HCl (pH 8.3). All samples were supplemented with 2U/µl RNasin (Promega). FCS was then carried out at ambient temperature. Translational diffusion times and the percentages of 2nd and 3rd components were calculated with the FCS ACCESS software (Version 1.0.12) using one- and three-component fit models as described previously (27).

Capillary Electrophoresis (CE). An automated HP3D Capillary Electrophoresis System (Hewlett Packard, Waldbronn, Germany) equipped with an uncoated fused-silica capillary was used throughout (27). Detector signals were recorded in fast spectral scanning mode to allow for monitoring at more than one wavelength. Positive polarity mode (negative pole placed at the capillary outlet) at 25 kV was used for all experiments. HRV-A2 was acidified by incubation in 50 mM NaOAc (pH 5.2) at 34°C for 15 min. To detect viral RNA outside the virion (but still connected to it), 1 µl (1 µg) anti-(ds)RNA MAb J2 (English & Scientific Consulting Kft) per sample (6 µg HRV-A2 in 19 µl) was added, followed by incubation for 20 min at room temperature. For detection of accessible poly-(A) tails, samples were incubated with poly-(U) (25 mer) at a final concentration of 0.5 µM for 10 min at room temperature prior to addition of MAb J2. All samples were run in 100 mM borate buffer (pH 8.3) containing 10 mM Thesit (Polyethylene glycol 400 dodecyl ether; [28]).

Extraction of RNA from the agarose gel and RT-qPCR. Native HRV-A2 and subviral particles resulting from acidification were incubated with micrococcal nuclease (MNase) at 37°C for 20 min and run on a 0.7% agarose gel in TEA buffer. Bands corresponding to intermediate subviral particles (whose accessible RNA had been digested) and native HRV-A2, respectively, were excised and RNA within the particles was extracted by using the “Zymoclean™ Gel RNA Recovery Kit” (ZymoResearch). Reverse transcription (RT) was carried
out with M-MLV reverse transcriptase (Promega) and followed by PCR for 30 cycles using
GoTaq® Hot Start Polymerase (Promega) and the primer sets indicated in the Figures and
used previously (27); qPCR was performed by using the KAPA SYBR Fast quantitative PCR kit
(PeqLab) in an Eppendorf Master Cycler. All samples were run in duplicate.

Uncoating in vivo. HeLa-Ohio cells grown in suspension were infected with HRV-A2 at an
MOI of ~1000. In control experiments, cells were preincubated at 34°C for 30 min with
niclosamide at 2µM and the drug was maintained at the same concentration throughout the
experiment. Virus was allowed to attach to cells in suspension at 4°C for 4 h with gentle
shaking. Unbound virus was removed by washing 2 times with cold PBS, the cells were
resuspended in 1 ml infection medium for suspension culture, and bound virus was allowed
to be internalized synchronously at 34°C for the times indicated in the Figures. The infected
cells were harvested via centrifugation, washed with PBS, resuspended in 500 µl PBS
containing 10 mM Theisit, broken with a Dounce homogeniser with a tight-fitting pestle, cell
debris was removed by low-speed centrifugation, and viral uncoating intermediates were
immunoprecipitated with MAb 2G2 (29) bound to magnetic protein-A beads (Dynabeads®
Protein A, Life Technologies); accessible RNA was digested with 10 U/sample of MNase at
room temperature for 10 min.

Cellular fractionation. Subcellular fractions (Fig. 5, left lanes) were prepared essentially as in
(30, 31). Briefly, HeLa-Ohio cells grown in suspension culture (5 x 10⁸) were collected,
washed with PBS, resuspended in 3 vol hypotonic buffer (1.5 mM MgCl₂, 10 mM KCl, 0.2 mM
PMSF, 0.5 M DTT, 10 mM HEPES (pH 7.9)) and allowed to swell on ice for 10 min. Cells were
broken in a Dounce homogenizer with 15 strokes using a loose-fitting pestle. Cell lysis was
monitored under the microscope. Debris, nuclei, mitochondria, and aggregates were
removed at 15,000 x g for 15 min and 0.11 vol 0.3 M HEPES (pH 7.9), 1.4 M KCl, 30 mM
MgCl₂, 0.2 mM PMSF and protease inhibitors pepstatin A and leupeptin hemisulphate salt
(at final concentrations of 2 µM and 0.6 µM, respectively) were added, and an aliquot
(termed ‘cyto’) was kept. The remainder was ultracentrifuged at 100,000 x g for 60 min at
4°C; the supernatant was termed ‘S100’. The pellet was resuspended in 50 mM Tris-HCl (pH
7.4) containing protease inhibitors as above (termed ‘P100’).
Preparation of microsomal fractions (Fig. 5, right lanes) was essentially as described in (32). HeLa-Ohio cells \((10^9)\) grown in suspension culture were collected at 1,200 rpm for 15 min in a Heraeus Megafuge 1.0, resuspended in 40 ml PBS, incubated for 10 min at room temperature in PBS containing 0.02% EDTA, and again pelleted. The resuspended pellet was washed twice with 150 mM NaCl, 1 mM Ca(OAc)\(_2\), 50 mM Tris-HCl (pH 7.4), once with 20 mM Tris-HCl, 10 mM EDTA (pH 7.4), cells were pelleted, resuspended in 5 ml Tris-EDTA buffer and incubated for 15 min at 4°C. The swollen cells were then broken in a Dounce homogenizer with 18 strokes using a tight-fitting plunger. Cell homogenization was monitored under the microscope. Larger debris, nuclei, and aggregates were removed by centrifugation in a table-top Eppendorf centrifuge at 900 \(x\) g for 5 min. The supernatants were transferred into SW40 centrifuge tubes and mixed with 75% sucrose (w/w) in 2 mM Tris-HCl buffer (pH 7.0) to give 50% final sucrose concentration. A step gradient was created by overlaying this dense solution with 2.7 ml 35% sucrose, 2.7 ml 30% sucrose, and 2 ml 25% sucrose (w/w) in the same buffer; finally 1 ml of plain buffer was applied and the tube was centrifuged in a SW40 rotor at 26,000 rpm for 17 h at 0°C. Three turbid bands were identified visually and collected from the top down in a total of 6 fractions (F1-F6). These were diluted approximately 1:3 with 50 mM Tris-HCl buffer (pH 7.0) and membranes were pelleted for 1 h at 0°C at 70,000 g using a TLS100 rotor (Beckman). The pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.0) containing 0.2 mM PMSF, 2 µM pepstatin A, and 0.6 µM leupeptin. The volumes were adjusted to contain roughly the same protein concentration as determined with a NanoDrop instrument from PeQLab (fraction 1 and 2 were very low in protein and used undiluted).

Western blotting and immunodetection of marker proteins. To localize early endosomes and lysosomes, aliquots were analyzed by western blotting with suitable antibodies. About 20 µg total protein of each fraction was separated on a 12% reducing polyacrylamide SDS-gel and transferred onto a polyvinylidene difluoride (PVDF) membrane using a Fast Semi-Dry blotter (Thermo Scientific). Membranes were blocked with 3% (w/v) BSA in PBS and processed for immunodetection with mouse anti-EEA1 (BD Transduction Laboratories) and mouse anti-LAMP1 (Developmental Studies Hybridoma), respectively. As a secondary antibody, goat anti-mouse-HRP (Jackson Laboratories) was used. EEA1 and LAMP1 were visualized via chemiluminescence (Supersignal, Invitrogen).
**In vitro RNA uncoating in the presence of cellular fractions.** Virus was mixed with the microsomal fractions at a final protein concentration of about 1 mg/ml in the presence of RNasin (4 U/µl, Promega). In the control sample the microsomal fraction was replaced with 50 mM Tris-HCl (pH 7). Additionally, aliquots of the fractions were preincubated in 10 mM Thesit for 15 min at room temperature for lysis of vesicles prior to mixing with virus. Samples were incubated at room temperature for 10 min, 1.5 vol 50 mM Na(OAc) (pH 5.2) was added and incubation continued at 34°C for 15 min. Samples were reneutralized via addition of the same vol of 100 mM Tris-HCl (pH 8.3). Where not yet present, 0.11 x vol 100 mM Thesit in 50 mM Tris-HCl buffer (pH 7) was added and the uncoating intermediates were collected via immunoprecipitation.

**Immunoprecipitation.** As a control for nonspecific RNase digestion, all samples were supplemented with 2 µg/µl Arabidopsis mRNA (a kind gift of A. Barta, Med. Univ. Vienna, Austria). Immunoprecipitation was carried out with MAb 2G2 and protein-A magnetic beads as above. To exclude contamination with viral nucleic acids, buffer alone was processed identically as a negative control. (Sub)viral particles in the immunoprecipitates were disintegrated with 200 µl 50 mM Tris-HCl (pH 7) containing 1% (w/v) SDS at 70°C for 5 min. Released viral RNA was then precipitated from the supernatant with ethanol in the presence of 1 µg/µl glycogen as a carrier and used for RT-PCR employing the M-oligo primer set (see above). In parallel, the integrity of the added control RNA was assessed by RT-PCR using primers specific for Arabidopsis ubiquitin (also generously provided by A. Barta). In control incubations with niclosamide, MAb 2G2 was replaced with rabbit polyclonal antiserum against HRV-A2.
RESULTS

Incubation of HRV-A2 at pH 5.2 results in exposure of 3’-terminal sequences of the genomic RNA, but 5’-proximal sequences remain protected inside the viral shell. HRV-A2 was incubated in NaOAc buffer (pH 5.2) (33-37) at 34°C, the optimal replication temperature of HRV, for 15 and 60 min and re-neutralized on ice. RNA sequences derived from the 3’ and 5’-ends, having eventually become accessible, were then separately determined with fluorescence correlation spectroscopy (FCS) as previously described for virus heated to 56°C (27). The FCS setup employed measures the diffusion of a fluorescent oligonucleotide; when such a probe hybridizes to cognate sequences present in free and/or incompletely-released RNA (still connected to the virion), its diffusion is slowed (reviewed in (38)); see Table 1 for the respective diffusion constants. The autocorrelation curves in Fig. 1A show that incubation at pH 5.2 followed by re-neutralization led to exposure of poly-(A) sequences present at the 3’-end at 15 min but not at 0 min (control). Extension of the incubation at acidic pH to 60 min did not change the signal appreciably, indicating that the number of subviral particles carrying accessible 3’-terminal sequences capable of hybridizing to the oligo-(dT) remained the same and that no free RNA was appearing. At neither time point were 5’-proximal sequences accessible to the cognate 5’-specific oligonucleotide (Fig. 1B). This can be deduced from the lack of a shift in the autocorrelation curve in Fig. 1B, indicating that the 5’-oligonucleotide was the only fluorescent component present; the respective components detected with this setup are indicated in the diagrams. The current results are strikingly different from those obtained when uncoating was triggered via heating; in this latter case, the entire RNA was released within about 20 min (27). These data imply that sequences close to the 5’-end of the viral genome remained inaccessible inside the capsid for at least up to 60 min of incubation at pH 5.2 at 34°C; therefore, no empty capsids were found (see below).

RNA exposed on acidification is recognized by dsRNA-specific antibodies only after hybridization with poly-(U). In previous work we had detected externalized RNA by using the dsRNA-specific monoclonal antibody (MAb) J2 (39); when HRV-A2 was subjected to psoralen cross-linking followed by heating, addition of this antibody led to a clear shift in migration of the generated subviral particles in capillary electrophoresis (CE). This was taken
to indicate that MAb J2 bound to partially-released RNA that had refolded into double-stranded regions (27). On repeating such experiments, but using acidification instead of heating for triggering the formation of subviral particles (as in Fig. 1), no change in the position of the broad peak of these ‘intermediate particles’ (i.e. A-particles with partially extruded RNA, denoted ‘I’) was observed in the presence of MAb J2 (compare Fig. 2 upper left panels); rather, the particles continued to migrate identically to the previously characterized intermediate particles (27, 40) and differently from native virus (‘N’). We thus reasoned that the exposed RNA segments might not fold into double strands long enough to be recognized by MAb J2. Furthermore, although poly-(A) can form double-strands at acidic pH, it fails to do so at neutral pH (41) and uridine-rich sequences suitable for base-pairing with the poly-(A) appear to be absent from the externalized sequences.

Poly-(A)/poly-(U) hybrids are recognized by MAb J2 (39); we thus added poly-(U) prior to incubation with the antibody. As seen in Fig. 2 (compare upper right panels), and in accordance with the literature (42), the added poly-(U) apparently hybridized to the externalized, and obviously accessible poly-(A) and gave rise to double strands that were now recognized by MAb J2; this resulted in a shift and broadening of the intermediate-particle peak. Together with the above FCS results, these results strongly suggest that exposure of HRV-A2 to acidic buffer leads to exit of the poly-(A) tail and presumably of upstream sequences with a low tendency to form double-stranded regions recognizable by J2. Thus, incubation of HRV-A2 at pH 5.2 initializes uncoating, but the RNA does not exit completely even after overnight incubation (data not shown); its continued egress requires cellular factors (see below). No shift in migration of the intermediate particles was seen when incubated with poly-(U) and an irrelevant antibody or when the virus-poly-(U)-J2 complex was treated with RNase.

Upon acidification in vitro, about 700 bases upstream of the 3’-end become exposed. To determine the extent of acid-triggered RNA release more precisely, we modified the RT-PCR protocol previously employed for the determination of sequences protected from RNase in psoralen UV-crosslinked and heated HRV-A2 (27). Virus was incubated for 0, 15, 30, and 60 min in pH 5.2 buffer at 34°C; the samples were re-neutralized and cooled to 4°C as above, accessible RNA was digested with MNase, and the resulting subviral particles were separated from the degradation products via agarose gel electrophoresis (Fig. 3A). Bands were excised,
the RNA extracted, and sequences having remained protected inside the capsid were quantified with RT-qPCR by using a graded set of roughly evenly-spaced oligonucleotide primer pairs (Fig. 3B). A cartoon illustrating the procedure is depicted in Fig. 3A. Fig. 3B shows that native, MNase-treated HRV-A2 gave rise to a band close to the top of the agarose gel whereas acidified, MNase-treated virus migrated further down. There was no obvious difference in the migration when incubation at pH 5.2 was for 15, 30, or 60 min. Apparently, despite the A-particle being expanded by 4% compared to the native virion (16), the RNA core is easily accessible for ions because of the various channels in the subviral particle shell (16), thus imparting on it a strong overall negative charge resulting in accelerated migration. Alternatively, loss of the charge-neutralizing polyamines (43) may explain this unexpected migration behavior. We assumed a copy number of 1 for all sequence stretches flanked with a primer pair in native virus. Nevertheless, as the band of HRV-A2 stained much more strongly with gel green (and Coomassie brilliant blue; not shown) than those of the subviral particles, we refrained from normalizing the data because of the obvious loss of material presumably due to aggregation impeding entry of part of the A-particles into the gel. Indeed, where acidified virus was applied, the gel pockets showed much stronger staining than in case of native virus (see Fig. 3B). For this reason, the data Fig. 3C is reported as relative, rather than absolute copy numbers of capsid-internal sequences along the genome as a function of incubation time at acidic pH. It is evident that 3’-proximal nucleotides quickly egressed on acidification and consequently rapidly disappeared upon subsequent RNase treatment. However, even at 60 min, sequences from the 5’ end extending to a region around position 6,500 were still protected. In conclusion, roughly 700 3’-proximal bases exit immediately, but further egress of the RNA molecule occurs extremely slowly, if at all.

In vivo, the entire viral genome is released within about 2 min. It was previously determined, using MAb 2G2 that specifically recognizes A- and B-particles but not native virus (44), that HRV-A2 converts into subviral particles in HeLa cells in less than 15 min (45). This includes entry and transport to late endosomes where pH 5.6, the threshold for structural modification (46) is attained. At 10 min, viral RNA accessible to a FISH probe can already be detected in the perinuclear area of the infected cells (47). We thus wanted to know how quickly the entire RNA is released from the virion in vivo. HeLa cells were challenged with virus at 34°C and entry/uncoating was halted via transferring the samples to...
ice at the timepoints given in Fig. 4. Cells were homogenized and subviral particles were
immunoprecipitated with MAb 2G2 to exclude background from residual native virus that is
not recognized by this antibody. RNA outside the virion was digested and specific RT-PCR
was carried out on the sequences that had remained protected from the nuclease inside the
capsid. As a control, niclosamide was added to the cells prior to viral challenge (Fig. 4, upper
right panel). This drug neutralizes the intra-endosomal pH, preventing conversion of native
virus into A-particles (48). As indicated in Fig. 4 (upper left panel), 3’-end proximal sequences
were detected at timepoints between 5 and 14 min post infection (pi), but were absent from
the subviral particles on infection times equal to or longer than 16 min. On the other hand,
5’-proximal sequences remained detectable for up to 16 min pi, but then also disappeared.
Thus, unlike the partial egress observed following acidification in vitro, RNA egress in vivo
grew to completion. Taking into account that RNA exit has just started as soon as 3’-terminal
sequences are no longer detected inside the viral shell and is complete when no 5’-terminal
sequences remain, the entire process takes about 2 min at 34°C. Note that no RNA was
detected at 0 min because native virus is not recognized by 2G2 and thus not
immunoprecipitated. Nevertheless, its presence can be inferred from the control experiment
in which polyclonal antiserum against HRV-A2 was used; these antibodies do not
discriminate between native virus and subviral particles (upper right panel). In this latter
experiment, 3’ and 5’ proximal sequences remained detectable for up to 60 min, the time of
observation. As expected, the virus thus remained intact in the presence of the drug.

When compared to the incomplete in vitro uncoating above, in vivo uncoating
completed in roughly 2 min. It appears to be initiated upon arrival of the virions in an
endosomal compartment with pH ≤ 5.6. Since trafficking from early to late endosomes (and
further to lysosomes) is strongly temperature-dependent, we also explored uncoating at
20°C and 25°C. As also seen in Fig. 4 (lower panels), viral RNA sequences were detected
much later at 25°C and even more so at 20°C. This temperature effect presumably results
from prolonged trafficking times that lead to delayed conversion of the virion into A-
particles and consequently of recognition by 2G2. Whereas viral RNA can be detected upon
infection at 34°C within roughly 5 min, it is seen only after 10 and 18 min at 25°C and 20°C,
respectively. Does uncoating still work at these lower temperatures? At 34°C, the 3’-end
becomes accessible to MNase between about 14 and 16 min, as seen from its absence at the
latter time point. At 25°C it did so between 30 and 60 min; finally, at 20°C it did not seem to
become externalized to an appreciable degree for up to 60 min when the experiment was terminated. Furthermore, at least for up to 60 min, 5’-proximal sequences remained protected at these lower temperatures whereas uncoating was complete in roughly 2 min at 34°C. Collectively, these experiments suggest that uncoating is easily initiated (i.e. the 3’ poly-(A) is still released at 25°C, although at a diminished speed), but the remainder of the genome is only extruded at higher temperatures and only in vivo.

Upon in vitro acidification in the presence of a microsomal fraction, RNA exit goes to completion. The large difference between in vitro and in vivo RNA release upon acidification suggests that cellular factors are required for the complete and rapid exit of the viral genome. Since RNA transfer from within the capsid into the cytosol occurs inside endosomes, such a factor might be expected to accumulate in a microsomal fraction. Microsomes isolated from homogenized cells include inside-in and, at a lower proportion, inside-out vesicles; the latter reform on closure of membrane sheets (49). Thus, such facilitators are expected to be accessible in cellular extracts. HeLa cells were homogenized and membranes were collected by high speed centrifugation. These were then fractionated by sucrose density step gradient centrifugation. Both crude subcellular fractions and microsome-enriched fractions were analyzed for their capacity to promote viral uncoating at low pH. The presence of the endosomal and lysosomal markers, early endosomal antigen 1 (EEA1) and lysosome associated membrane protein 1 (LAMP1), respectively, was also assessed. Virus was incubated with the respective membrane fractions, acidified, and the extent of RNA release was estimated after immunoprecipitation with MAb 2G2. To control for nonspecific degradation, irrelevant Arabidopsis mRNA, including sequences encoding ubiquitin, was added to all samples. RNA extracted from the immunoprecipitates and RNA in the supernatants was tested for the presence of 5’-proximal sequences by RT-PCR as above.

As seen in Fig. 5, except when acidified in the presence of the resuspended membrane pellet (V+P100; for preparation of the membrane fractions see M&M) and membrane fractions 4 and 5 (F4 and F5) from the sucrose density step gradient, the 5’-proximal sequences of the viral RNA remained detectable inside the virion, indicating a lack of complete uncoating. In the remaining samples, no RNA was detected, implying complete release and/or degradation. Except in the sample incubated in the presence of membrane fraction 5, nonspecific degradation could be definitely excluded, because the control RNA was intact.
In all supernatants of the immunoprecipitates, viral RNA with intact 5’-sequences was found (middle panel); it was most probably derived from remaining intact virus (compare to the in vitro experiment in Fig. 2). Therefore, fraction 4 appears to contain a factor which promotes exit of the entire viral RNA genome on acidification.

**DISCUSSION**

The recent determination of the 3D structures of three enterovirus empty capsids to near atomic resolution (16, 25, 50) revealed important details of the conformational rearrangement occurring on transition of the native virion into the subviral intermediate of RNA uncoating. The native virion expands, loses VP4 and externalizes N-terminal segments of VP1. As shown in the present report, it also externalizes the poly-(A) tail together with several hundred upstream nucleotides. This stretch corresponds to roughly 10% of the total RNA, a number similar to an earlier estimate of about 7%, based on the UV absorbance ratios at 200 nm and 260 nm of the peaks of native virus and RNase-treated intermediate-particles in CE (51).

Most likely, similar to poliovirus (17), the RNA exits through one of the channels close to the two-fold axes in a timely, coordinated fashion to allow for its efficient transfer into the cytosol for replication. Egress of the RNA is believed to occur after the A-particle dissociates from the receptor (52); concomitantly, the particle might be handed over to the endosomal membrane where the amphipathic N-terminal segments of VP1, and presumably VP4 set free during the process, insert into the membrane for its destabilization and pore formation (37, 53-55). Our observation that the poly-(A) stretch already exits upon acidification adds a new dimension to the uncoating process; the 3’-end of the RNA might be actively participating in the correct positioning of the viral particle onto the inner endosomal membrane, thereby initiating and directing its transfer. Although the ratio between physical particles and infectious particles was estimated to be on the order of 24 up to 6500 (56-58), it seems counterintuitive that the viral genome would exit randomly with 29 chances in 30 (there are 30 symmetry-related holes in the subviral particle) to end up in the lumen of the (late) endosome where it might be degraded.

It did not come as a great surprise that the 3’ poly-(A) exits first (27), since not being involved in the formation of double strands might facilitate sampling the inner face of the capsid for the presence of holes. Alternatively, because of its peculiar features it might...
become positioned, already during assembly, close to a two-fold axis where a pore is to open on conversion of the native virion into the subviral A-particle. No *Enterovirus* counterpart to the maturation protein present as a single copy in the capsid of MS2 phage (59) has been found. Such proteins had been overlooked previously because of the icosahedral averaging intrinsic to X-ray crystallography and most often employed in cryo-EM 3D-image reconstruction to increase the resolution. It is possible that asymmetry is introduced in *Enteroviruses* by the presence of non-capsid proteins associated in spurious amounts with the shell, such as those involved in replication that were detected in highly purified foot-and-mouth disease virus, another picornavirus (60). Small amounts of VP0 have also been noticed in purified *Enteroviruses* and it has not been clarified whether they stem exclusively from natural empty capsids, the ‘natural top component’ (35) or from individual virions containing a small proportion of VP0 that might establish asymmetry. It is conceivable that the presence of a few VP0 molecules could break the icosahedral symmetry thus favouring RNA exit at a specific (presumably membrane-juxtaposed) location.

The difference between *in vitro* and *in vivo* uncoating is particularly striking. At least in the case of minor group HRVs it cannot be explained by a ‘catalytic’ function of the receptor; binding of a very-low density lipoprotein receptor mimetic, a head-to-tail concatamer of ligand-binding repeat V3 that exhibits high affinity for HRV-A2 (61) stabilizes the virus rather than aiding in its conversion to A-particles (62). This is dissimilar to major group viruses which are uncoated by an excess of soluble ICAM-1 alone (63). Furthermore, the presence of lipid membranes alone seems to assist RNA exit only marginally (36). The uncoating activity we found in the microsomal membrane fraction of HeLa cells is preserved after extensive dialysis but abrogated by detergents (not shown). This might suggest that the process is energy independent but relies on intact membranes. Coxsackie virus B3 uncoating activity has been observed in membrane extracts previously (32, 64). Its receptor DAF does not have uncoating activity but its second receptor CAR has, which might explain this finding (65). Ongoing studies in our laboratory are aimed at characterizing the “HRV-A2 uncoating facilitator” identified in HeLa cell membrane fractions.
Acknowledgments

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Figure Legends

Fig. 1 Sequences at the 3’-end of the viral RNA, including the poly-(A) tail, become accessible upon acidification of native HRV-A2 at 34°C. Normalized autocorrelation curves of A) DyLight 488-labeled oligo-(dT) incubated with native virus (black), and with virus incubated at pH 5.2 for 15 min (red) and for 60 min (blue). B) As in A) but in the presence of an oligonucleotide complementary to a sequence close to the 5’-end (443-468). The cartoons show the components present in the incubation mixtures and their interactions; note the absence of free RNA and of sequences recognized by the 5’-specific oligonucleotide at all timepoints.

Fig. 2 RNA sequences externalized on incubation of HRV-A2 at pH 5.2 are not recognized by ds-specific MAb J2 as shown by capillary electrophoresis. Acidified or native virus, as indicated, were incubated with the specified components and analyzed by capillary electrophoresis. The subviral particle exposing RNA (‘intermediate particle’) migrates as a broad peak at a position different from that of the A-particle (see also ref. (27)). Upon removal of the exposed RNA sequences with RNase, the particle co-migrates with the A-particle. Note (i), addition of poly-(U) together with MAb J2, but neither one alone, modified the migration of intermediate particles. Note (ii), replacement of MAb J2 with an irrelevant antibody (anti-γ−tubulin) at the same concentration had no effect on the migration of viral or subviral particles. Note (iii), native virus did not change its migration on addition of poly-(U) together with MAb J2 demonstrating the absence of accessible poly-(A) sequences. Internal standard, DMSO (is). Broken vertical lines indicate the position of the ‘intermediate particles’ (I). Electropherograms were calibrated to the migration of DMSO and, where present, of RNasin (R). N, native virus; A, A-particle.
Fig. 3 At pH 5.2, HRV-A2 releases an RNA segment including about seven hundred residues at the 3'-end. A) Cartoon illustrating the procedure; virus was incubated in acidic buffer at 34°C for the times indicated, re-neutralized, and accessible RNA was digested. B) Native HRV-A2 (control) and the subviral particles resulting from the above treatment were separated on a 0.7% agarose gel. RNA within the particles was visualized with gel green. DNA markers were run in the leftmost lane. C) RNA was extracted from the bands in B) and the presence of sequences was assessed with RT-qPCR using primer pairs hybridizing to the indicated positions in the viral genome. The number of copies is given as an arbitrary number. The approximate trend of the time-dependent loss of RNA sequences (from 3' to 5') is indicated by a trend plot.

Fig. 4 In vivo release of viral RNA is rapid. HeLa cells (~10^6) grown in suspension culture were suspended in 1 ml infection medium and HRV-A2 (MOI ~ 1,000) was allowed to attach at 4°C for 4 h, cells were washed with PBS, resuspended in fresh infection medium and incubated for the indicated times. Cells were lysed, intermediate particles were collected via immunoprecipitation with 2G2, externalized RNA was digested with MNase, and protected sequences were identified with RT-PCR using the primer pairs complementary to the viral genome at the positions indicated at the left. In the upper right panel (control), uncoating was inhibited via addition of niclosamide (Nic), present during the whole experiment, and immunoprecipitation was with polyclonal rabbit antiserum directed against HRV-A2 (αHRV-A2). Experiments were carried out at the temperatures indicated under otherwise identical conditions. The status of the virion and of its RNA is shown as a cartoon below the gels; black line, time of conversion into A-particle; blue line, time until the 3'-end becomes accessible from the A-particle; green line, time until complete RNA release. N, native; A, A-particle; I, intermediate particle (with partially released RNA); E, empty capsid.

Fig. 5 HeLa microsomal fraction promotes RNA uncoating. HRV-A2 was acidified in the presence of subcellular fractions (left) or of microsomal fractions obtained from a sucrose density step gradient (right). Upper panel) Subviral particles were immunoprecipitated with MAb 2G2, RNA was extracted from the immunoprecipitates and analyzed for the presence of 5'-proximal sequences via RT-PCR using primer pair M followed by 1% agarose gel...
electrophoresis and gel green staining. To exclude loss of the RNA via digestion by contaminating RNases, the supernatant was again immunoprecipitated, but now with MAb 8F5 (66) to remove any remaining native virus; RNA was then extracted from the supernatant and analyzed as above. As a control, an irrelevant RNA (ARA; Arabidopsis mRNA containing ubiquitin-encoding sequences) was added to all samples prior to immunoprecipitation and RNA extraction and was detected with a ubiquitin-specific primer set. Fractions framed were positive for LAMP1 and EEA1 as determined via western blotting. Lower bands are from the primers. To exclude contamination of the reaction mixture with viral RNA, RT-PCR was carried out without the addition of viral material (negative control). Shown is one out of 5 independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>3’oligo (dT)</th>
<th>5’oligo</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>D(10^-12)m^2/s</td>
<td>%</td>
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<tr>
<td>oligo+Virus-Ac (60min)</td>
<td>9.9 ± 0.6</td>
<td>77.4 ± 2.1</td>
</tr>
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Table 1 Diffusion coefficients of labeled 5’-oligonucleotide and oligo-(dT) upon binding to free viral RNA and to RNA connected to the virion, respectively. Diffusion coefficients of the free oligonucleotide and the corresponding second component after incubation with virus and acidification for different time periods, as well as the corresponding fractions of those components, are given. Standard deviations were calculated from 3 independent experiments in which each measurement was taken between 10 and 30 times. Diffusion coefficients were derived according to equation 1 by fitting the obtained autocorrelation curves, g(t), to the model.

\[ g(t) = \frac{1}{N} \sum_{i=1}^{M} \frac{f_i}{(1+t/\tau_{Di})(1+t/\kappa \tau_{Di})^{1/2}} \]  

(1)

Where N is the total number of fluorescent particles, f_i the fraction of each component present at equilibrium and \( \tau_{Di} \) the respective diffusion coefficients, \( \kappa \) the structural parameter, and M the number of distinct components. In the FCS experiments described
here the components were either $M = 1$ or $M = 2$ (in those cases where the percentage is indicated).

References


65. Milstone AM, Petrrella J, Sanchez MD, Mahmud M, Whitbeck JC, Bergelson JM. 2005. Interaction with coxsackievirus and adenovirus receptor, but not with decay-

A

- Oligo3+Virus
- Oligo3+Virus-Ac(15min)
- Oligo3+Virus-Ac(60min)

B

- Oligo5+Virus
- Oligo5+Virus-Ac(15min)
- Oligo5+Virus-Ac(60min)

Normalized Autocorrelation

Correlation Time [ms]