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Research Article

Characterization of rhinovirus subviral A particles via capillary electrophoresis, electron microscopy and gas-phase electrophoretic mobility molecular analysis: Part I

During infection, enteroviruses, such as human rhinoviruses (HRVs), convert from the native, infective form with a sedimentation coefficient of 150S to empty subviral particles sedimenting at 80S (B particles). B particles lack viral capsid protein 4 (VP4) and the single-stranded RNA genome. On the way to this end stage, a metastable intermediate particle is observed in the cell early after infection. This subviral A particle still contains the RNA but lacks VP4 and sediments at 135S. Native (150S) HRV serotype 2 (HRV2) as well as its empty (80S) capsid have been well characterized by capillary electrophoresis. In the present paper, we demonstrate separation of at least two forms of subviral A particles on the midway between native virions and empty 80S capsids by CE. For one of these intermediates, we established a reproducible way for its preparation and characterized this particle in terms of its electrophoretic mobility and its appearance in transmission electron microscopy (TEM). Furthermore, the conversion of this intermediate to 80S particles was investigated. Gas-phase electrophoretic mobility molecular analysis (GEMMA) yielded additional insights into sample composition. More data on particle characterization including its protein composition and RNA content (for unambiguous identification of the detected intermediate as subviral A particle) will be presented in the second part of the publication.

Keywords:
Capillary electrophoresis / Electron microscopy / GEMMA / HRV2 / Subviral particle
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1 Introduction

Human rhinoviruses (HRVs) are the main causative agents of the common cold. Apart from their phylogenetic classification into species A, B, and C, they are separated into a major and a minor group according to receptor specificity; major group viruses (more than 90 serotypes) bind intracellular adhesion molecule 1 (ICAM 1), minor group viruses (12 serotypes) bind members of the low-density lipoprotein receptor (LDLR) family (for a recent review refer to [1]). The cellular receptor of HRV-Cs is unknown. HRVs are nonenveloped and of icosahedral symmetry with a diameter of 30 nm. Sixty copies each of the four viral proteins (VP1–VP4) form the viral capsid and enclose a single-stranded RNA genome of approximately 7.1 kb in length.

In early investigations on infection by minor group HRV serotype 2 (HRV2), Lonberg-Holm and Yin [2] observed, in addition to the native virion, two different forms of subviral particles sedimenting in sucrose density gradients at 135S and 80S, respectively. These particles were also obtained in vitro; incubation of infectious virions with acidic buffer (conditions resembling the in vivo environment in endosomes) yielded predominantly 135S particles, whereas almost exclusively 80S particles were produced on incubation for 5 min at 56°C or on incubation with 2 M urea in PBS for 60 min at 22°C. Whereas both subviral particles lack VP4,

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Abbreviations: **DMA**, differential mobility analyzer; **EMD**, electrophoretic mobility diameter; **GEMMA**, gas-phase electrophoretic mobility molecular analysis; **HRV**, human rhinovirus; **HRV2**, HRV serotype 2; **TEM,** transmission electron microscopy; **VP,** viral protein

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135S particles still contain the viral RNA. They are thus believed to represent an intermediate of the uncoating (i.e. RNA release) process. This was also suggested by the finding that 135S particles of the closely related poliovirus are infective although to a greatly reduced rate when compared to native virions [3].

In vivo, HRV2 conversion takes place upon clathrin-dependent receptor-mediated endocytosis [4]. Virions are internalized in coated pits, shuttled to early endosomes and further to late endosomes where they become exposed to pH values lower than 5.6; this is the threshold pH for virus uncoating [5]. Subsequently, or concomitantly, with the conformational changes of the virus capsid the viral RNA genome is transferred through the endosomal membrane into the cytosol of the infected cell by yet not fully known mechanisms.

Capillary electrophoresis (CE) is a valuable tool in the analysis of biomolecules allowing separation of particles in solution according to their different migration properties upon application of an electric field. We and others have introduced CE into the field of viral analysis and quality control of viral preparations [6–11]. For HRV2, capillary isoelectric focusing allowed for determination of its pI to a value of 6.8 [12]; later we demonstrated separation of viral (150S) and subviral 80S particles of HRV2 by CE [13]. Since the acid sensitivity of HRV2 precluded the electrophoretic separation in acidic buffers, and in order to obtain reasonable values for the electroosmotic flow and thus relatively short CE migration times, analyses were carried out in large part in sodium borate at pH 8.3 as background electrolyte (BGE). However, aggregation and adsorption to the bare silica capillary walls were still problematic and could only be remedied by switching to electrophoresis (of fluorescence-labeled virus particles) in the chip format [14, 15] or by using detergent additives such as SDS or Thesit, employed above their respective critical micellar concentration (CMC). This latter greatly reduced the number of spikes recorded in the capillary format and improved the reproducibility of particle separation [16]. These adjustments finally allowed to analyze interactions between virus and soluble recombinant receptor fragments [17–19], antibodies and antibody fragments [18, 20, 21], as well as investigation of the influence of antiviral substances on viral uncoating [22]. CE is now being employed routinely in our laboratory to assess the quality and concentration of HRV preparations. However, detection of subviral 135S particles of HRV2 by CE was only reported once [22] and not further pursued. Therefore, in the present report, we concentrated on the analysis and characterization of this metastable form of the virion appearing during conversion of native virus to the empty capsid by a combination of CE, transmission electron microscopy (TEM), and gas phase electrophoretic mobility molecular analysis (GEMMA). The utility of GEMMA for the analysis of human rhinovirus and its complexes with recombinant receptor fragments and antibodies has been demonstrated previously [23, 24]. It is based on the separation of single charged particles in an electric field of a differential mobility analyzer (DMA). Single charged particles are obtained via a nano electrospray process. This is a so-called "soft ionization" technique known not to fragment large protein assemblies – for recent reviews refer to [25–27] – followed by solvent evaporation and charge reduction in a bipolar atmosphere. Particles from such a nano electrospray charge-reducing source are introduced into the DMA unit via a sheath flow of CO_2 and air. Simultaneously, an orthogonal electric field is applied, which allows for separation of monodisperse particles according to different electrophoretic mobility diameters (EMD) by voltage scanning. Monodisperse particles from the DMA corresponding to a given separation voltage and hence a certain EMD are introduced to the condensation particle counter (CPC) unit of the instrument where they act as condensation nuclei for droplet formation in a supersaturated 1-butanol atmosphere. Other than the current CE setup with UV absorption or fluorescence detection, droplets are detected by light scattering of a focused laser beam independent of the chemical composition. Therefore, GEMMA yields valuable additional information on particle EMD values of analytes.

As can be learned from our results, the combination of several unrelated analysis methods allows for a more in-depth characterization of the content of a viral sample. In our case, CE of HRV2 exposed to low pH or 56°C demonstrates the existence of at least two intermediate subviral particles identical or related to the rhinoviral 135S particles. Furthermore, extending our investigations to GEMMA and TEM analyses, a first characterization of a contamination found in all our HRV2 preparations at variable concentrations was possible.

2 Materials and methods

2.1 Chemicals and reagents

Boric acid (99.99%), SDS (reagent plus grade), ammonium acetate (≥99.99%), and acetic acid were from Sigma Aldrich (Steinheim, Germany), sodium hydroxide (pellets pure), sodium acetate, benzoic acid, and 25% ammonia in water (all pro analysis, p.a.) were from Merck (Darmstadt, Germany). Tris (ultrapure) was from AppliChem (Darmstadt), Thesit (polyethylene glycol dodecyl ether, membrane research grade) and DMSO (p.a.) were from Fluka (Buchs, Switzerland). Thyroglobulin from bovine thyroid gland (≥ 90%) used as internal standard in GEMMA measurements was from Calbiochem (Darmstadt). Phosphotungstic acid, 2% in water, pH 7.3 was employed as negative stain for TEM imaging. Water was bidistilled from a quartz apparatus and of Millipore grade (18.2 MΩcm resistivity at 25°C). HRV2 was prepared and its purity and concentration assessed as described [13, 28].

In short, HRV2 was grown in HeLa cells in suspension culture. Infected cells were broken via repeated freezing/thawing cycles and cell debris was removed via centrifugation. Virions were pelleted from the supernatant and treated with RNase, DNase (both 10 min at ambient temperature), Trypsin (5 min at 37°C), and N-laurylsarcosine (over night at 4 °C) after resuspension of the pellet in virus preparation buffer A (VPA; 20 mM Tris-HCl, pH 7.5, including

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Table 1. Electrophoretic net mobilities, μ, of native virus and subviral particles

Particle	μ[a)]	SD[a)]	*t*	*n*
Contaminant[b)]	−12.5	0.3	20	36
Contaminant[c)] (heat)	−7.1	0.3	25	21
	−6.6	0.2	20	21
Contaminant[c)] (pH)	−7.2	0.1	25	21
	−6.7	0.2	20	21
Native 150S[b)]	−22.2	0.3	20	36
Native 150S[c)]	Same as contaminant[c)]; compare to 5.7 from ref. [16]			
135 S[c),d)]	−16.6	0.4	20	5
Intermediate AI[c)] (heat)	−19.6	0.5	25	21
	−17.1	0.4	20	21
Intermediate AI[c)] (pH)	−19.3	0.3	25	21
	−17.3	0.4	20	21
Intermediate AII[c)]	−13.0	1.4	20	11
Subviral 80S[c)] (heat)	−9.5	0.2	25	21
	−8.2	0.3	20	21
	Compare to 8.5 from ref. [16]			
Subviral 80S[c)] (pH)	−9.6	0.1	25	21
	−8.5	0.3	20	21

Heat—sample incubated at 56°C for 10 min. pH—sample incubated at pH 5.0 for 15 min. μ averaged from *n* measurements. t: measuring temperature (°C); SD: standard deviation; *n*: number of measurements.
a) [10^{-9} m^2/Vs]
b) 100 mM sodium borate (pH 8.3), 10 mM SDS
c) 100 mM sodium borate (pH 8.3), 10 mM Thesit.
d) Preparation according to ref. [2]

2 mM magnesium chloride). Again debris was removed via centrifugation and virus from the supernatant purified via sucrose gradient centrifugation (7.5–45% sucrose in VPA). Fractions including HRV2 were collected and virus was pelleted over night prior to resuspension of virions in 50 mM sodium borate, pH 7.4. Aliquots from several HRV2 batches were employed in the present publication with typical virus concentrations in the range of several mg/mL (around 0.5 μM) as determined via CE.

2.2 Buffers

BGE for CE separations was 100 mM sodium borate, pH 8.3, containing 10 mM Thesit (BGE). Electrophoretic mobility values were also determined in SDS at 10 mM in the case that particles were stable under these conditions (see Table 1). Sample buffer (SB) was BGE without surfactant. BGE and SB were filtered (cellulose acetate (CA) membrane, 0.20-μm pore size syringe filters, Sartorius, Göttingen, Germany) and centrifuged (1 min at 800 rcf on a tabletop centrifuge, 5415D from Eppendorf, Hamburg, Germany) prior to use. Sodium acetate (NaOAc) buffer was 50 mM (pH 5.0). For use as GEMMA electrolyte, 25 mM ammonium acetate (NH_4OAc) (pH 7.4) was filtered through an surfactant-free CA membrane syringe filter (0.20-μm pore size, Sartorius).

2.3 Instrumentation

CE was carried out on 3D CE instruments (Agilent, Waldbronn, Germany) employing fused silica capillaries (Polymicro, Phoenix, AZ, USA) of 50 μm id, 375 μm od, and an overall length of 59.9 ± 0.1 cm. The effective length for analyte detection via UV absorption at indicated wavelengths was 51.4 ± 0.1 cm. Electrophoresis was performed in positive polarity mode at 25 kV (approximately 42 kV/m field strength) at specified temperatures. Samples were at ambient temperature and introduced into the capillary by application of pressure as indicated. Preconditioning was by flushing with BGE for 2 min, postconditioning by flushing with 1 M sodium hydroxide (2 min), and water (2 min).

GEMMA measurements were carried out on an instrument consisting of an electrospray aerosol generator (model 3480), an electrostatic classifier (series 3080) with a nano DMA unit, and an ultrafine CPC (model 3025 A) from TSI Inc. (Shoreview, MN, USA). Cone tip capillaries with 40 μm inner, 150 μm outer diameter, and 24 cm length (TSI Inc.) were employed. GEMMA settings were 0.1 liters per minute (Lpm) carbon dioxide and 1.0 Lpm air sheath flow for the nano electrospray process, 15.0 Lpm sheath flow were applied for particle separation in the DMA unit, and 4.4 psid (pounds per square inch differential, approximately 30 kPa) pressure difference across the capillary for sample introduction. Voltage for the electrospray process was 1.9 kV resulting in a current of approximately 370 nA. GEMMA spectra were recorded in the range of 3–50 nm EMD (110 s scantime, 20 s reset time). Median values of ten spectra each are depicted (individual spectra are not shown). Buffer exchange for GEMMA measurements was done on a tabletop centrifuge (1–14 from Sigma, Osterode am Harz, Germany) for 6.5 min at 9.30×10^3 rcf, respectively (see below).

TEM negative stain imaging was on a FEI Morgagni instrument (FEI Tecnai, Eindhoven, The Netherlands) as described [29] at the indicated magnifications. Pelleting was in a Beckman L7 ultracentrifuge at 1.55×10^5 rcf for 1 hour at 4°C.

2.4 Sample preparation

Conversion of native HRV2 to subviral particles was via incubation in a water bath for 10 min at 56°C if not indicated otherwise (due to small sample volumes, the elevated temperature was reached almost immediately after immersion); alternatively, acidification by addition of NaOAc for 15 min at ambient temperature unless otherwise noted was employed for virus conversion. Volumes required for obtaining respective pH values were tested in large scale and verified with

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a pH electrode. For comparison with 135S particles as described in the literature, native HRV2 was incubated with 1 M NaOAc, pH 5.0, for 20 min at ambient temperature followed by reneutralization via addition of 0.5 M Tris base according to [2]. Buffer exchange against NH_4OAc for GEMMA measurements was carried out with spin filters (polyethersulfone (PES) membrane, 10 kDa cutoff from VWR, Vienna, Austria). The HRV2 stock solution was diluted 1:262 (v/v) in NH_4OAc solution and 50 μL of this dilution were weighted to 480 μL NH_4OAc on the top of the spin filter. After centrifugation, the eluate was removed, 500 μL NH_4OAc replenished and spinning repeated for a second round. NH_4OAc (60 μL) was applied to the filter to retrieve retained material. Subsequently, the filter was washed with another 60 μL aliquot of NH_4OAc. Retrieved aliquots were combined, weighted, and brought to a 1:5 (v/v) dilution according to the initially weighted material (adjustment with NH_4OAc assuming a buffer density of 1 mg/μL). Final dilutions were with NH_4OAc including Thyroglobulin (as a marker) to obtain samples at 0.5 nM HRV2 and 10.0 nM Thyroglobulin, respectively. Pelleting of the contaminant (sample component first detected upon CE of HRV2 [16]) was found possible for one HRV2 preparation batch and was carried out after incubation of 5 μL HRV2 stock with 15 μL NaOAc for 15 min at ambient temperature followed by addition of 980 μL SB. The contaminant precipitated under these conditions was pelleted and resuspended in SB prior to further sample processing for CE, TEM, and GEMMA measurements. For CE runs, HRV2 concentrations were typically in the range of 0.05 μM, benzoic acid (as internal standard; IS) was at 0.02 mg/mL, and DMSO (as EOF marker) was diluted in the range of 1:3.4 × 10^3 (v/v) in SB.

3 Results and discussion

CE of purified HRV2 in sodium borate (100 mM, pH 8.3) containing 10 mM Thesit (note that the CMC is 0.1 mM in water at 20–25°C according to the manufacturer) yields only one peak from two unresolved sample constituents – a contaminant present in the virus preparation and the native virus itself [16]. After incubation of the sample for 10 min at 56°C, native virus is converted to subviral 80S particles that lack VP4 as well as the viral RNA genome [30]. Both species, intact virus and subviral 80S particles can be separated despite the very small difference in their net electrophoretic mobility, μ: for native virus (and the contaminant) μ is around -7×10^{-9} m^2/Vs, and for 80S particles around -9×10^{-9} m^2/Vs (see Table 1 for a summary of the electrophoretic parameters). The presence of RNA in intact virions is reflected in a UV absorption maximum at 260 nm [22]. Figure 1 depicts electropherograms and the corresponding negative stain TEM images of intact virus (A and E) and of empty capsids (D and G). Native virions appear as bright spheres, whereas subviral 80S particles show a bright circle with a core of high density due to their permeability for the stain that accumulates within the viral shell. Areas of small, bright spots were sometimes found in preparations of subviral 80S particles (Fig. 1G, circled area); these might result from virions denatured during the staining process.

In the course of endocytosis, subviral 135S particles (A particles) represent a metastable state of the virion. In these particles, amphipathic N-terminal sequences of VP1 are externalized; in vivo, during infection, they insert into the lipid bilayer of endosomes. It is believed that these protein domains, together with the myristoylated, hydrophobic VP4 form a channel through the lipid bilayer for RNA transfer. For HRVs, so far only one intermediate form of viral uncoating has been described after sucrose density gradient velocity centrifugation and immunodiffusion analysis [2]. However, under the conditions used here for subviral particle formation (see below) two forms of intermediate particles, separable by CE (Fig. 1B) were found; we termed them AI and AII. The presence of free RNA in these preparations let us to assume that AI and AII possess the same capsid conformation but are distinct in their RNA content or genome accessibility. They might represent particles with partially released or degraded RNA. The occurrence of several types of subviral particles has previously been reported only for poliovirus that had been characterized by ultracentrifugation in sucrose density gradients [31, 32].

A striking difference between the two rhinoviral intermediates AI and AII is their stability; intermediate AII particles (μ is -13.0×10^{-9} m^2/Vs at 20°C, Table 1) completely converted into empty capsids on keeping them over night at room temperature or on incubation for 2.5 min at 56°C. For intermediate AI particles (μ around -17.2×10^{-9} m^2/Vs at 20°C, Table 1), neither treatment led to an appreciable reduction of the peak area or to modified CE migration. In fact, the electropherogram in Fig. 1C was obtained after heating the sample – whose CE trace is depicted in Fig. 1B – for 2.5 min to 56°C. For the present publication, we further concentrated on the characterization of the more stable intermediate AI form. Reanalysis of a corresponding sample even after several days storage at 4°C showed that only about 20% of intermediate AI were converted to empty 80S particles (data not shown).

It is of note that formation of AII particles was highly dependent on the individual virus preparation (exact mechanisms of AII stabilization are still unclear), even more pronounced than for AI particles (see below). Therefore, we focused on virus preparation batches predominantly yielding AI particles upon virus conversion (the majority of investigated batches) for further experiments.

3.1 Characterization of intermediate AI particles

A sample yielding an electropherogram as in Fig. 1C showed particles in negative stain TEM (Fig. 1F) that are easily distinguishable from native virus (Fig. 1E) and empty 80S capsids (Fig. 1G). Pickl-Herk et al. (manuscript in preparation) demonstrated expansion of the capsid diameter by 4% on conversion from native virus to 135S and further to 80S particles.

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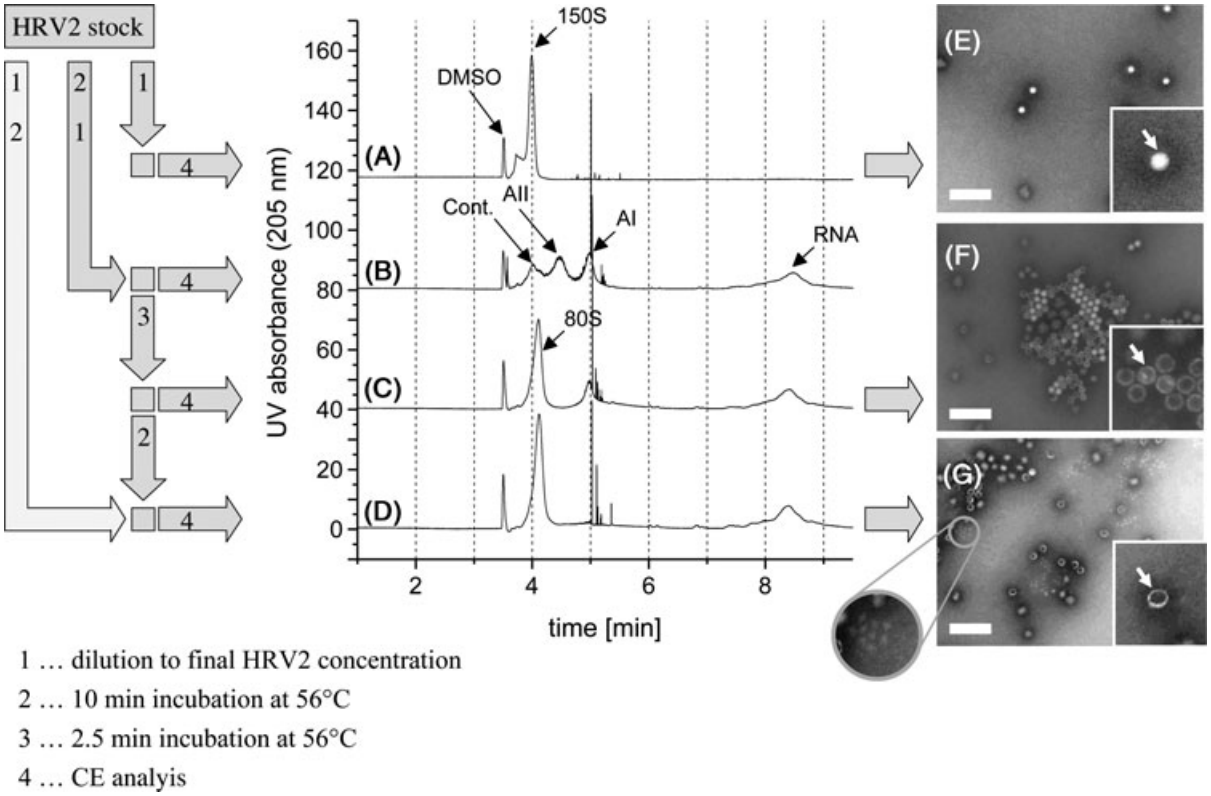

Figure 1. Two different subviral A particles are formed at 56°C. (A) CE trace of intact (150S) virions; (B) intermediate AI and AII particles; (C) AI particles and subviral 80S particles; (D) subviral 80S particles. The particles in (C) were obtained by heating particles in (B) for 2.5 min at 56 °C. The particles in (D) were obtained by heating particles in (C) for another 10 min at 56°C. A schematic overview of preparation of samples is also given with the figure. Clear shifts for corresponding peaks are observed upon virus conversion. The series of electropherograms also demonstrates an increase of the signal corresponding to the RNA released upon progression of uncoating. Uncoated fused silica capillary, 50 μm id, L_{tot} = 60.0 cm, L_{eff} = 51.5 cm; Voltage: 25 kV in positive polarity mode (approximately 42 kV/m field strength) at 20°C. BGE included 10 mM Thesit. Injection: 50 mbar pressure for 9 s. Detection at 205 nm, obtained signals are in mAU. HRV2 was at approximately 0.05 μM in SB with DMSO at 1:4.0 × 10^3 [v/v]. Subviral particle samples had about the same concentrations but were in half concentrated SB. Electropherograms of the subviral particles were corrected for some slight shift in EOF by aligning the DMSO signals. TEM image (E) corresponds to CE trace (A) (native virions), TEM image (F) to CE trace, (C) and TEM image (G) to CE trace (D) (subviral 80S particles). Note that TEM image (F) is not restricted to one particle population — as a result of intermediate particle preparation, particles corresponding to beginning and end points of virus conversion are also represented. Samples were stained with 2% sodium phosphotungstate (pH 7.2) and viewed at 8.9 × 10^4 fold magnification. The size bars correspond to 200 nm. Insets depict representative particles (marked by arrows) at about threefold higher magnification than large images. Note irregularly shaped particles, e.g. at the bottom of (E), for comparison with Fig. 6C.

Furthermore, VP4 is lost. Both result in more space for the RNA allowing for its relaxation; in addition, holes open in the capsid that permit stain penetration. This explains the grainy texture of the interior of A particles. The images are in accordance with recently published data [29] that showed similar intermediate particles upon pH-triggered virus uncoating at the surface of liposomes. In this latter paper, various forms of subviral particles were observed that most probably correspond to virions having released RNA to a different degree. Similarly, in Fig. 1F particles presumably containing condensed fragments of RNA are seen (arrow) together with particles with grainy but homogenous interior; these latter most probably have not yet initiated RNA exit. Therefore, we assumed that two different populations of subviral particles as observed via TEM are reflected by two intermediate forms as resolved by CE.

As mentioned above, subviral particles are obtained in vitro by exposure to low pH but also when being kept at 56°C for several minutes. The former procedure was reported to predominantly yield 135S particles, the latter predominantly empty 80S particles [2]. It is of note that 135S particles prepared according to ref. [2] exhibited an electrophoretic net mobility $\mu = -16.6 \times 10^{-9}$ m^2/Vs (Table 1) in CE, a value in good accordance with the electrophoretic net mobility of AI particles as stated above. However, our initial experiments demonstrated that incubation at 56°C for 10 min (Fig. 1B) as well as acidification (Fig. 2) generated AI particles. In order to induce the conformational change by low pH, native HRV2 was mixed with NaOAc buffer to yield solutions of pH values between 6.0 and 5.0 (in increments of 0.2 units). As a control, virus was incubated under otherwise identical conditions at neutral pH. In fact, already at a pH of 5.8, native

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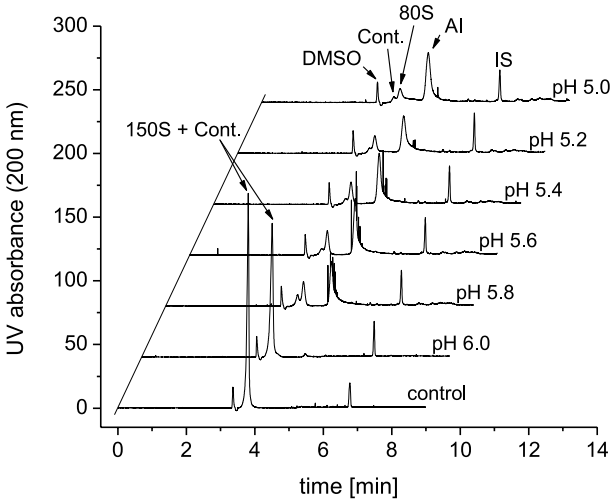

Figure 2. Electropherograms of virus particles upon uncoating as function of the pH. Subvirus particles formed by incubation at different pH values (from 5.0 to 7.4) by addition of NaOAc buffer. Injection was at 50 mbar for 8 s and electrophoresis as in Fig. 1 at 25°C. DMSO and benzoic acid were used as neutral marker and internal standard, respectively. Detection was via UV absorption at 200 nm, obtained signals are in mAU.

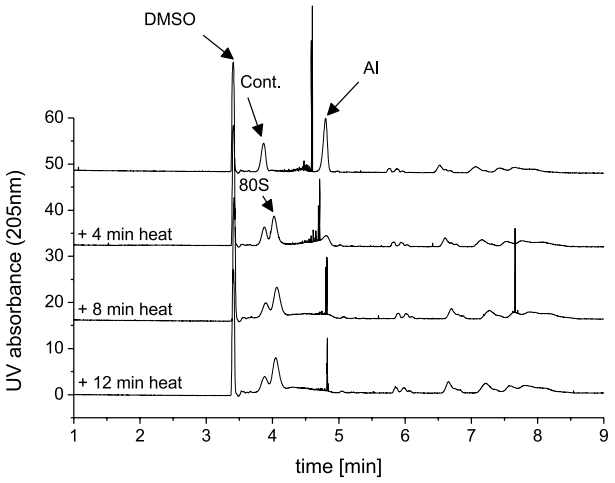

Figure 3. Electropherograms of intermediate AI particles after incubation at 56°C for various time intervals. A sample containing AI particles (generated by 10 min incubation at 56°C) was further incubated at the same temperature for indicated times. Note the complete conversion of AI particles to subviral 80S particles at a total time of 12 min. CE conditions as in Fig. 1 with the exception of $L_{tot} = 59.8$ cm, $L_{eff} = 51.3$ cm. Virus was 0.04 μM in SB including DMSO at 1:2.1 × 10^3 [v/v] dilution.

virions were almost completely converted to AI intermediates (Fig. 2). This pH value is slightly higher than that published for the formation of subviral 135S particles, but the small divergence might be accounted for by differences in ionic strength and buffer composition. However, as can be seen from Table 1, electrophoretic net mobilities for AI particles were independent of whether uncoating was triggered by acidification or heating. Therefore, although acidification is more similar to the conditions found during in vivo infection, exposure to 56°C was employed in the following for the preparation of AI particles because of simpler handling of the samples and the avoidance of dilution.

Are AI particles "true" intermediates of viral uncoating or just represent a dead end, i.e. correspond to abortive infection? Application of further triggers of viral uncoating via additional sample heating to 56°C (4, 8, and 12 min) revealed that AI particles (generated via HRV2 heating to 56°C for 10 min) were capable of complete conversion to 80S particles (Fig. 3). The decrease of the AI peak resulted in a concomitant increase of the peak of 80S particles. As long as AI particles were present, spikes were recorded that probably resulted from aggregation of hydrophobic particles; native virus and 80S particles are hydrophilic.

Overall, we concluded that AI and AII particles might represent virions with their RNA being released to different degrees; consequently, to complete the release, they appear to require different times. Since only two and not a continuum of forms of the A particle were identified, it is possible that their RNA adopts different conformations. Alternatively, there might be a stop and go mechanism of RNA release similar to the one described for the DNA phage λ [33].

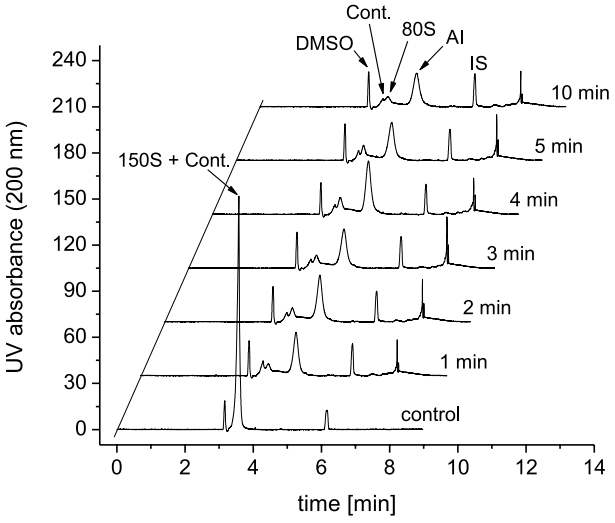

Figure 4. Electropherograms of subviral particles generated on incubation at pH 5.0 as function of time. Experimental conditions as in Fig. 2.

Remarkably, adjustment of the pH to between pH 5.0 and 6.0 (Fig. 2) or incubation at pH 5.0 for up to 10 min (Fig. 4) did not result in a full conversion of AI into 80S particles. Only a small fraction of native virus was converted to 80S particles by acidification (compare, e.g. to Fig. 2, trace pH 5.0). Therefore, it appears that low pH alone is insufficient to trigger conversion of AI to 80S particles and consequently for RNA extrusion. From Fig. 4 it becomes apparent that about three-fourth of the native virions remain AI particles and do not convert further, unless being heated. It is thus likely that AI particles are trapped at a local energy minimum

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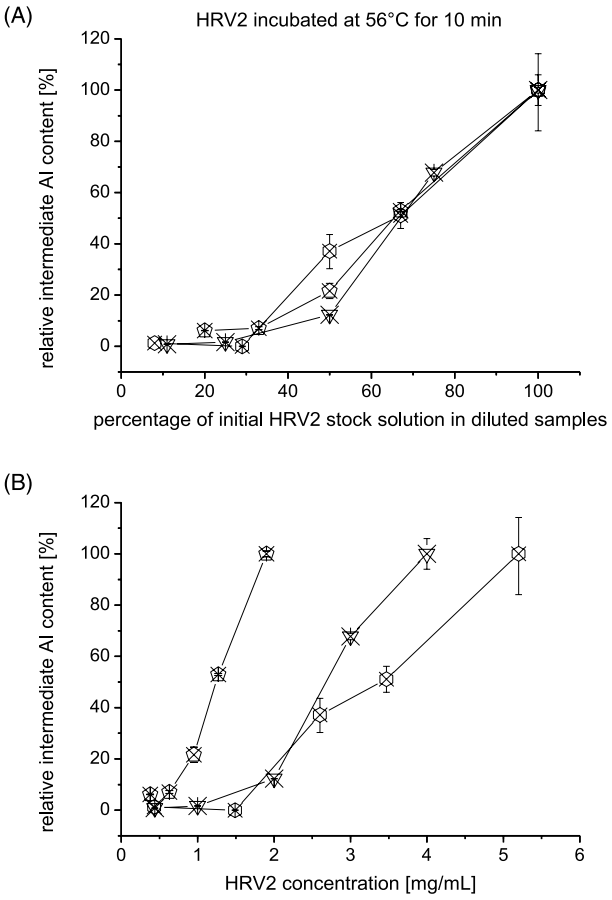

Figure 5. Relative intermediate AI particle sample content upon heat conversion of native virus at 56°C; data obtained from three independent HRV2 preparations relative to the percentage of initial HRV2 stock solution in diluted samples upon heat-induced virus conversion are plotted (A). The same data, but relative to the HRV2 concentration is shown in (B). Note the clear correlation between the percentage of initial HRV2 stock solution in diluted samples and AI particles present in the sample after incubation at 56°C. Error bars indicate the range for at least duplicate measurements.

but are fully capable to undergo further conversion (as can be deduced from the results presented in Fig. 3). Lipid membranes, as present during infection of the host cell or even another compound of the extracellular matrix, might be necessary for conversion of A particles to empty capsids at the low endosomal pH.

3.2 Parameters influencing formation of intermediate AI particles

We found that the extent of formation of AI particles was highly dependent on the individual virus preparation. Even more surprising, we noticed that the degree of dilution of the virus stock solution prior to triggering conversion at 56°C (for 10 min) was decisive for the yield of AI particles rather than the virus concentration. When the virus stock solutions were diluted only weakly, they gave high yields of AI particles. Higher dilutions mainly led to the formation of 80S

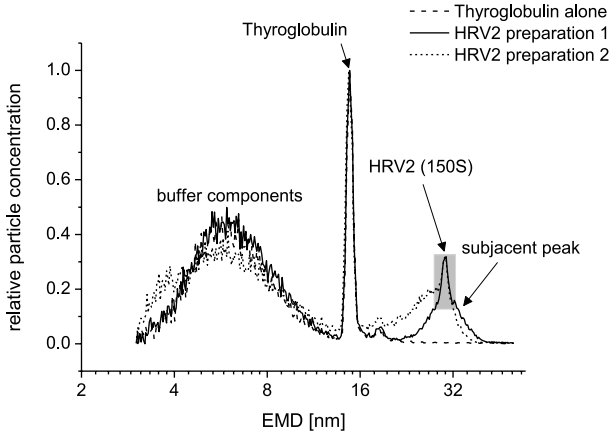

Figure 6. Detection of a contaminating component present in the virus samples. GEMMA spectra of HRV2 samples after buffer exchange to NH_4OAc (25 mM ammonium acetate, pH 7.4). Results for two independent HRV2 preparations are presented; virus was at approximately 0.5 nM. Samples additionally included approximately 10 nM Thyroglobulin as internal standard. Besides a relatively sharp peak for intact virions at 30.1 nm EMD (highlighted), a second subjacent broad peak is detected. Particles with up to 15 nm EMD correspond to aggregates of buffer components (from the electrospray process). GEMMA spectra were recorded upon application of a cone tipped capillary (40 μm inner diameter, L_{tot} = 24.0 cm) and 0.1 Lpm CO_2/1.0 Lpm air sheath flow at approximately 1.9 kV/–370 nA current nanospray conditions. DMA sheath flow was at 15 Lpm. Median values of ten spectra each are depicted (110 s scantime, 20 s retrace time per scan) after relation to the peak of the internal standard.

particles (Fig. 5A). As a consequence, conversion of native HRV2 could be directed to give rise preferentially to intermediate AI or B particles by simply adjusting the degree of virus dilution. However, most astonishingly, whereas the degree of dilution of the initial virus preparation was well correlated with the fraction of AI particles obtained, no correlation was found with the concentration of the virus in the samples; a preparation with low virus concentration (and without dilution) yielded AI particles, whereas in a sample of the same virus concentration (obtained upon dilution of a more concentrated virus stock), AI particles were no longer found (Fig. 5B). Therefore, we reasoned that an unknown component might be present in the preparation and would be responsible for the stabilization of the intermediate particles. This assumption is supported by the finding that subviral A particles of a related coxsackievirus are stabilized by a "saline-soluble factor" derived from a HeLa cell plasma membrane extract [34].

3.3 GEMMA of viral preparations demonstrates the presence of an additional sample component

GEMMA spectra of two viral preparations are shown in Fig. 6. Two EMD ranges related to the peak of Thyroglobulin as internal standard at 15 nm can be distinguished. The lower EMD range (between 3 and 15 nm) shows a broad signal originating from buffer components. The high EMD

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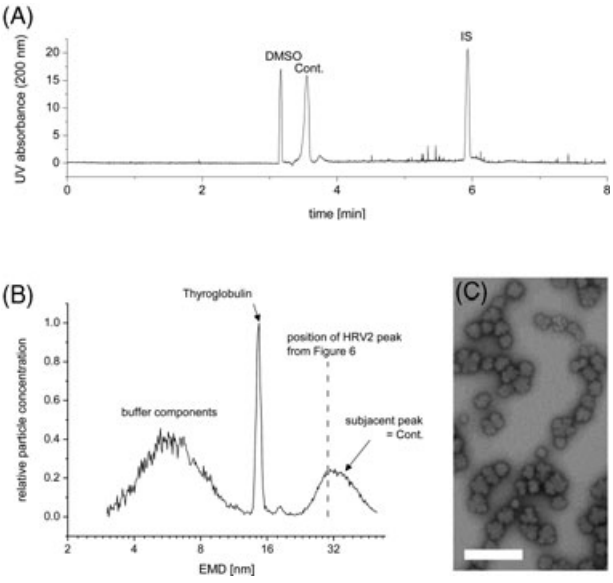

Figure 7. Correlation of the broad subjacent peak (GEMMA) with CE and TEM results. Isolation of the contaminating material (contaminant) always found at varying concentrations in virus preparations as first detected via CE was found possible for one HRV2 batch allowing for direct comparison of a respective sample via CE and GEMMA measurements as well as TEM imaging. Electropherogram (A) of the sample solely contained contaminant. The contaminant corresponds to the broad subjacent peak as found with GEMMA spectra (B); The EMD of HRV2 particles (compare to Fig. 6 for a sample still containing virions) is indicated in the spectrum by a dashed line. TEM image (C) of the contaminant; the contaminant is visualized as irregularly shaped particles. Electrophoretic analysis conditions as in Fig. 2, GEMMA conditions as in Fig. 6, TEM conditions as in Fig. 1. The image was viewed at 3.6×10^4 fold magnification.

range spanning from roughly 15–50 nm is relevant for analysis of HRV2. Two peaks can be detected; a relatively sharp one with a maximum at 30.1 nm ($\pm$ 1.5%) reflects very well the size of HRV2 particles as found previously by GEMMA [24]. Whereas the EMD of this peak was virtually identical for all HRV2 preparations analyzed ($n = 6$), the second, exceptionally broad peak varied greatly between different virus batches. It started at around 23 ± 2 nm EMD with its trailing edge at 43 ± 7 nm. Further experiments showed that this broad subjacent peak corresponded to contaminating material (contaminant) always found at varying concentrations in our virus preparations, as first detected by CE [16]. From one of the virus preparations, it was possible to specifically precipitate and pellet the contaminant allowing for its analysis with CE (Fig. 7A), GEMMA (Fig. 7B) and TEM (Fig. 7C). Therefore, correlation of the broad subjacent GEMMA peak to the contaminant recorded by CE and to the irregularly shaped particles upon TEM imaging of HRV2 (as found previously [29, 35]) was possible. Based on shape, overall size and preliminary data from enzymatic hydrolysis, we believe that the contaminant corresponds to membrane fragments, possibly exosomes of HRV2-producing HeLa cells. However, more experiments are required for its definitive identification. So far it is unclear whether this material or even another factor is playing a role in the formation of AI particles.

4 Concluding remarks

We were able to separate, by CE, two different subviral particles derived from HRV2 (that we termed AI and AII) representing intermediates of virus uncoating in vitro. We found that these intermediates are greatly differing in their stability and concentrated further on the characterization of the more stable form, AI particles, apparently closely related or identical with 135S (subviral A particles) of HRV2 (see also results of the second part of the publication). AII particles were found to be more readily transformed into empty capsids and might differ from AI particles in their RNA content or genome accessibility. Alternatively, we hypothesized that AII particles are competent for release of the complete RNA genome whereas AI particles might contain RNA with nicks. Also, the exiting nucleic acid molecule might have been cleaved during exit by contaminating RNases; such particles would remain after the leading part (it is unknown whether this is the 3′ or the 5′ end) of the RNA has left the capsid; the RNA fragment staying behind might not be able to reinitiate exit.

AI particles have an electrophoretic net mobility of -17.2×10^{-9} m^2/Vs and are produced on incubation of native virus for 10 min at 56°C as well as at pH values equal to or under 5.8. Particles obtained under both conditions were indistinguishable in terms of their electrophoretic mobility. However, further conversion of AI particles to empty (80S) capsids was only possible through incubation at 56°C. Acidification to pH values as low as 5.0, even for 10 min, failed to convert AI into empty capsids. Seemingly, these intermediates are fully capable of further conversion but are trapped in a local energy minimum. Under in vivo conditions, the presence of cell membranes or other cellular components might be required for complete RNA release.

We found that the ratio between AI and B particles produced from HRV2 was strongly influenced by experimental conditions; it was highly dependent on the dilution of the virus stock solution, but surprisingly virtually independent of the virus concentration. This led us to hypothesize that viral preparations contained an additional component impacting on the formation of subviral particles. Indeed, GEMMA analysis detected a highly heterogeneous material contaminating HRV2 preparations whose size was within that of virions. It could be related to the "contaminant" invariably seen by CE in our virus preparations. Furthermore, it corresponds to irregularly shaped particles often observed by negative stain TEM of virus samples. Preliminary data led us to assume that the contaminant originates from cell membrane fragments. Further experiments are necessary for unambiguous identification (see second part of the publication).

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