Human rhinovirus HRV14 uncoats from early endosomes in the presence of bafilomycin

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Abstract Determination of infectious progeny virus and in vivo labelling with [^{35}S]methionine followed by immunoprecipitation demonstrates that the major receptor group human rhinovirus HRV14 is able to infect HeLa cells in the presence of the V-ATPase inhibitor bafilomycin A1. However, host cell shut off is delayed and viral yield is decreased in the presence of the drug. Uncoating can thus take place under conditions that prevent endosomal acidification indicating that it is catalysed by the viral receptor alone. Since transport is arrested in early endosomes upon inhibition of vesicle acidification, the data also suggest that productive uncoating takes place from early endocytic compartments.

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Key words: Human rhinovirus; Viral uncoating; Bafilomycin; Endocytosis

1. Introduction

Human rhinoviruses (HRVs) are small RNA-viruses constituting a separate *genus* within the family *picornaviridae*. Based on the receptor used for cell entry, two groups have been defined [1]. The major group uses intercellular adhesion molecule-1 (ICAM-1) as a receptor while the minor group gains access to the host cell via members of the low-density lipoprotein receptor (LDLR) family [2–4]. The rhinovirus capsid can adopt three conformational states. Native virus sediments at 150S; A-particles are formed upon interaction with the host cell, have lost the capsid protein VP4, and sediment with 125–135S. These subviral particles are considered to be either a transition state in the uncoating pathway or a product of abortive infection. Finally, B-particles, sedimenting at 80S, remain after the release of the genomic RNA. The conformational changes represent a major step in viral entry and are clearly triggered by the host cell. In vitro, rhinoviruses are converted to A-particles upon exposure to low pH and to B-particles by heating to 56°C [5]. For major receptor group viruses, structural modifications to 135S or to 80S particles also occur upon interaction with recombinant soluble ICAM-1 in a strongly temperature-dependent manner [6,7].

We have shown previously that uncoating of HRV2, a minor group virus, requires uptake into late endosomes or endosomal carrier vesicles for uncoating to occur [8–10]. RNA release strictly depends on the low endosomal pH with the receptor being used only as a vehicle for cell entry. In contrast to HRV2, cellular uptake of HRV14 is slow and less efficient [11] possibly because ICAM-1 lacks typical coated pit localisation signals which are present in recycling receptors such as LDLR [12,13]. Nevertheless, some degradation of HRV14 starting at 60 min p.i. is clearly indicative of endocytosis and transport to lysosomes [11]. Moreover, in previous work we have explicitly demonstrated viral internalisation into endosomes by free-flow electrophoretic analysis of homogenates prepared from HeLa cells infected with HRV14 [14]. Since all rhinoviruses are acid labile, it has been commonly thought that uncoating requires uptake into acidic endosomal compartments [15–18]. However, the ICAM-1-induced conformational change in vitro proceeds at neutral pH without any synergistic effect of mildly acid pH [7]. This suggests that the 'catalytic activity' of the receptor might be sufficient for uncoating in vivo as well. Nevertheless, Perez and Carrasco reported that inhibition of endosome acidification resulted in lack of host cell shut off upon challenge with HRV14 as seen by continued incorporation of [^{35}S]methionine into cellular protein. This led them to conclude that HRV14 infection was dependent on the acidic pH in the endosome lumen [18]. However, in the course of a study aimed at comparing the mode of penetration of HRV2 and HRV14 into the cytosol we recently observed that viral protein was being synthesised upon challenge of HeLa cells with HRV14 even under conditions where endosome acidification was blocked [14].

In the present study we confirm and extend these results by demonstrating that infectious progeny virus is produced upon infection of HeLa cells in the presence of bafilomycin. However, the yield of infectious virus was found to be substantially decreased when compared to control infections. In vivo labelling of cellular proteins in the presence of the drug demonstrated a delay in host cell shut off, a reason why viral protein synthesis might have escaped detection in a previous investigation [18].

2. Materials and methods

2.1. Chemicals

All chemicals and tissue culture media were purchased from Sigma unless stated otherwise. Bafilomycin A1 (a kind gift of Prof. Dr. K.H. Altendorf) was dissolved in dimethylsulfoxide at 20 mM and stored at −20°C. The final concentration of the solvent in the incubation mixtures was kept below 1%. [^{35}S]methionine/cysteine (Translabel) was from American Radiolabeled Chemicals.

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Abbreviations: HRV, human rhinovirus; MOI, multiplicity of infection; $TCID_{50}$, 50% tissue culture infectious dose; p.i., post infection

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2.2. In vivo labelling of HRV14 and immunoprecipitation

HRV14 and HRV2 (originally obtained from ATCC) were propagated in HeLa cells (Wisconsin strain) and purified as described [19]. Cells were grown in suspension in S-MEM-Joklik with 7% horse serum. Virus was labelled as described by Neubauer and colleagues [15]. For the detection of de novo synthesised viral proteins, cells (2×10^5) were preincubated in 1.5 ml plastic tubes by slow rotation for 30 min in a water bath at 34°C in 500 μl of methionine-free MEM containing 2% foetal calf serum (dialysed against PBS) and 30 mM $MgCl_2$ (infection medium) in the absence or presence of bafilomycin, at the final concentrations as stated in the text. Cells were then challenged with the respective virus (suspended in 100 μl PBS) at an MOI of 500. At selected time points, the medium was replaced by fresh methionine-free infection medium (±drug) containing 20 μCi [^{35}S]methionine and incubation was continued for the times specified. Cells were pelleted, boiled in Laemmli sample buffer and separated on SDS-12.5% polyacrylamide minigels. Detection by fluorography was with Kodak XomatR films. Alternatively, cells were lysed in 300 μl RIPA buffer [20] and viral proteins were immunoprecipitated with rabbit anti-HRV14 antiserum bound to IgG-Sorb (Enzyme Centre) and analysed as above.

2.3. Determination of infectious progeny virus

HeLa cells grown in 24-well plates were preincubated with or without 400 nM bafilomycin in 200 μl infection medium for 30 min. One hundred MOI of HRV2 and HRV14, respectively, were added in 200 μl of the same medium resulting in a final concentration of bafilomycin of 200 nM. After the specified incubation times, plates were subjected to three freeze/thaw cycles to release cell associated virus, cell debris was removed by low speed centrifugation and the $TCID_{50}$ of the virus in the supernatants was determined accordingly. To those samples that had not received bafilomycin, drug was added prior to $TCID_{50}$ determination.

3. Results

3.1. Bafilomycin A1 fails to inhibit infection with HRV14

HeLa cells were preincubated in the absence or in the presence of bafilomycin at the final concentrations as shown in Fig. 1 and infected with HRV14 at an MOI of 500. At 4.5 h p.i. [^{35}S]methionine was added to label de novo synthesised viral proteins. At 19.5 h p.i. progeny virus was recovered from cell lysates by immunoprecipitation and viral capsid proteins were detected upon separation on an SDS-polyacrylamide gel followed by fluorography (for experimental set-up see top of Fig. 1). When compared to the control experiment (carried out in the absence of bafilomycin, Fig. 1A, lane 1) it becomes apparent that the continuous presence of the drug at final concentrations ranging from 200 nM to 2 μM failed to prevent viral infection as viral capsid proteins appeared almost equally labelled regardless of the presence of bafilomycin. As we have recently shown that bafilomycin at >200 nM efficiently raises the endocytic vesicle pH to neutrality in HeLa cells [10] this suggests that viral uncoating must have occurred at neutral pH. In order to investigate whether virus mediated host cell shut off (i.e. a decline in translation of capped cellular messenger RNAs due to cleavage of the initiation factor eIF-4G by the viral proteinase 2A [21]) was affected by the drug, total cell lysates were also analysed without immunoprecipitation (Fig. 1B). As expected, in the absence of bafilomycin (lane 2), viral challenge led to a substantial decrease of cellular protein synthesis when compared to mock infected cells (lane 1). However, no host cell shut off had occurred in the presence of 2 μM bafilomycin (compare lanes 2 and 3) at 4.5 h p.i. Moreover, in accordance with earlier results [9], protein synthesis per se is not affected by bafilomycin (compare lanes 1 and 3). This suggests that viral replication was delayed in the presence of the drug resulting in a lag of virally induced shut off of host cell protein synthesis. When viral progeny released from the infected cells and recovered from the cell supernatant by immunoprecipitation, the amount of viral proteins synthesised in presence of bafilomycin appeared unaffected as compared to infection in the absence of the drug (Fig. 1C). These data clearly establish that HRV14 infection can occur independently of low endosomal pH and that release of virus from the cells does not appear to be affected by the drug.

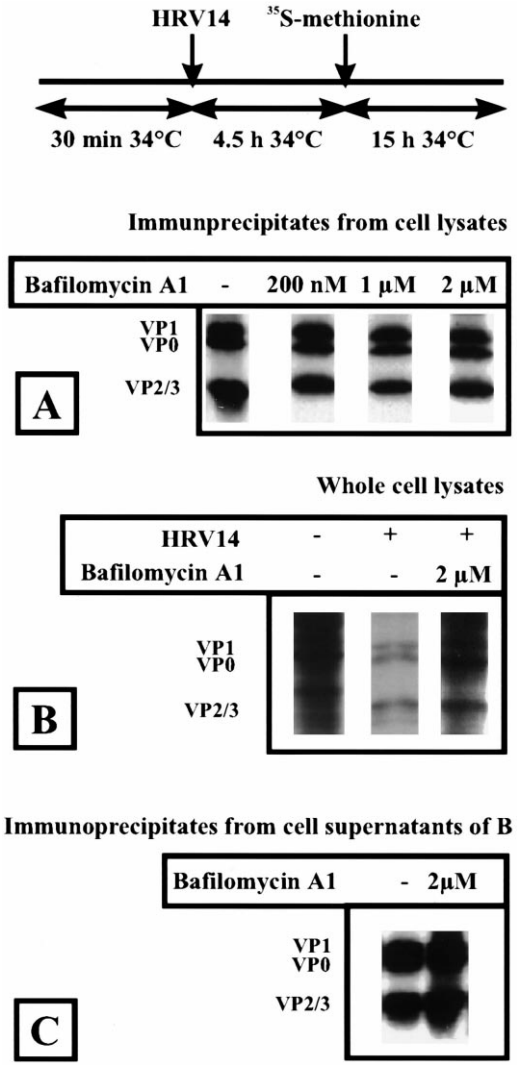


Fig. 1. Effect of bafilomycin on viral infection. HeLa cells were preincubated in the presence or absence of bafilomycin, infected with HRV14 and [^{35}S]methionine was added (for experimental set-up see top). (A) The cell pellet was solubilised, viral proteins were immunoprecipitated and separated by SDS-PAGE followed by fluorography. For comparison, (B) total protein in the cell pellet or (C) proteins immunoprecipitated from the cell supernatant were also analysed.

The lack of host cell shut off at 4.5 h p.i. in the presence of 2 μM bafilomycin is in accordance with results of Perez and Carrasco [18]. However, these workers interpreted their data as inhibition of infection due to elevation of endosomal pH. The immunoprecipitation as used in our experiments clearly demonstrates de novo synthesis of HRV14 in the presence of the drug, excluding the requirement for low intravesicular pH during infection.

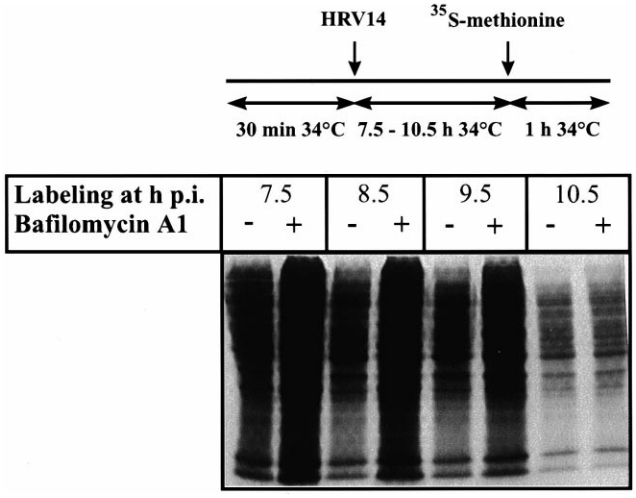


Fig. 2. Delay of virus-induced host cell shut off in the presence of bafilomycin. Cells were preincubated in the absence and presence of bafilomycin, infected, and de novo synthesised proteins were labelled for 1 h with [^{35}S]methionine at the time points p.i. as indicated. Analysis of total cellular protein was by SDS-PAGE followed by autoradiography. For experimental set-up see top of the figure. Where indicated, bafilomycin was present at 2 μM.

In order to investigate whether host cell shut off did not occur under our experimental conditions due to a delay in the onset of viral protein synthesis, total proteins made in virus infected cells were monitored by pulse labelling for 1 h at the times indicated in Fig. 2. The data of this experiment demonstrate that the peak of viral synthesis indeed occurs with a delay of about 2 h in the presence of 2 μM bafilomycin. As a consequence, host cell shut off is also delayed explaining the apparent discrepancy between our data shown in Fig. 1A and the results of Perez and Carrasco [18].

3.2. *Infectious HRV14 is synthesised in the presence of bafilomycin*

Incorporation of radioactivity into viral protein does not necessarily result in production of infectious virions. Therefore, the influence of bafilomycin on the synthesis of infectious virus was assayed by means of $TCID_{50}$ determination. For control purposes, the minor group rhinovirus HRV2 was also included in the analysis as we have previously demonstrated that bafilomycin completely blocked incorporation of [^{35}S]methionine into capsid proteins of this serotype [9].

HeLa cells grown in 24-well plates were preincubated with or without 400 nM bafilomycin for 30 min. Cells were then challenged with 1.6×10^8 $TCID_{50}$ HRV2 and HRV14, respectively, corresponding to an MOI of 100 (Fig. 3). Where indicated, bafilomycin was then maintained at a final concentration of 200 nM during the whole experiment. One h p.i. the supernatants containing virus that had not entered the cells were removed, cells were washed and incubation was continued in fresh infection medium (± 200 nM bafilomycin). Plates were subjected to three freeze/thaw cycles at the times p.i. as shown in Fig. 3. Samples without drug were adjusted to 200 nM bafilomycin prior to $TCID_{50}$ determination. In the absence of the drug, HRV2 infection led to a substantial increase of infectious virus at 20.5 h p.i. when compared to that present at 1 h p.i. or at 4 h p.i. The latter time point was chosen to determine the fraction of input virus that had not undergone uncoating and was thus still present as infectious particles. However, the increase in viral titre from 1 to 4 h p.i. indicates some de novo synthesis of progeny virus already at this early time point. In the presence of bafilomycin, the titre of HRV2 was about one log higher at 1 h p.i. when compared to the value in the absence of bafilomycin. Furthermore, this value did not change up to 20.5 h p.i. This is taken to indicate that all virus having entered the cells during the challenge period (1 h) remained in a native state and had not undergone uncoating or degradation in lysosomes. As bafilomycin does not affect virus uptake [9] one can assume that the same number of infectious particles had entered the cells regardless of the presence of the drug. Therefore, about 90% of internalised HRV2 must have been uncoated in the absence of the drug. In accordance with earlier results [9,15] these data thus clearly confirmed that HRV2 synthesis was completely blocked in the presence of the drug.

For HRV14, in absence of bafilomycin, the time-dependent increase of viral titre was very similar to that of HRV2. Input virus recovered at 1 h p.i. was also substantially higher in presence of the drug when compared to the value seen in its absence. However, at 4 h p.i. viral titre dropped by a factor of 1000 indicating that uncoating had taken place. Finally, the increase of viral titre at 20.5 h p.i. proves that viral replication occurs in the presence of bafilomycin.

Taken together, our results imply that the receptor alone is

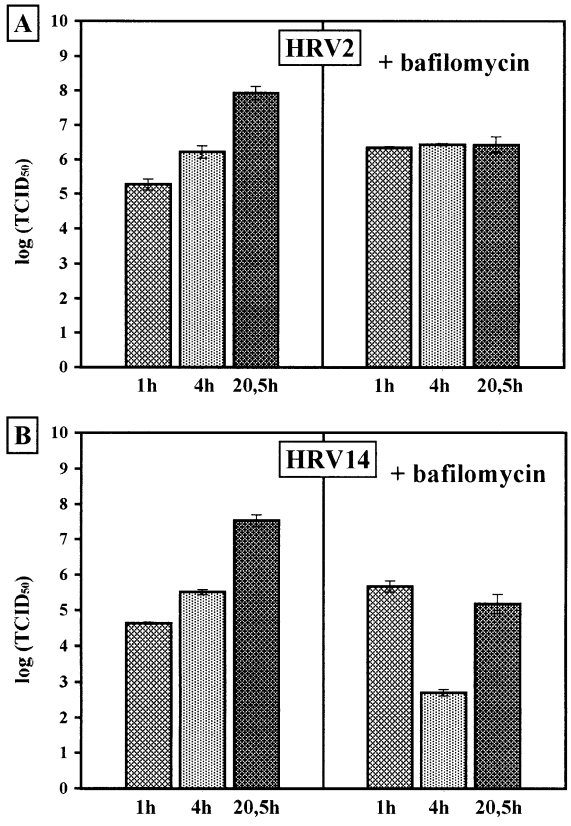


Fig. 3. Infectious HRV14 is produced in the presence of bafilomycin. HeLa cells grown in 24-well plates were preincubated with bafilomycin for 30 min at 34°C and infected with HRV2 or with HRV14 at an MOI of 100. Where indicated, the drug was then present throughout the experiment at a final concentration of 200 nM. At the times specified, viral titre was determined as $TCID_{50}$. Data represent the mean ± S.D. from three independent experiments.

capable of inducing the structural modification of the viral capsid required to initiate RNA release and productive infection even in the absence of a transmembrane proton gradient. Nevertheless, whereas HRV2 replication is clearly prevented by the presence of bafilomycin, uncoating of HRV14 proceeds but synthesis of infectious HRV14 is substantially diminished.

4. Discussion

Using immunoprecipitation of viral proteins we demonstrated that HRV14 infection of HeLa cells also occurs when endosome acidification is prevented by bafilomycin A1, a specific inhibitor of vacuolar H^+-ATPases. This is in sharp contrast to HRV2 infection, which is completely blocked by the same drug (this paper and [8,9]). HRV14 uncoating thus appears to be catalysed by its receptor alone under these conditions. Since host cell shut off is retarded in the presence of the drug (Fig. 2), incorporation of radioactive methionine into viral proteins might have been missed in previous work [18].

When the synthesis of radioactively labelled viral proteins (Fig. 1) was compared to the synthesis of infectious virions (Fig. 3) in the presence of the drug it became apparent that the increase in viral titre of HRV14 was much less than that expected from the radioactivity incorporated. The reason for this discrepancy is not clear at the present time. The higher MOI used in the in vivo labelling experiment (500 and 100, respectively) might override to some extent the delay in host cell shut off. Control experiments (not shown) demonstrated that addition of bafilomycin after viral challenge (1 h p.i.) neither significantly inhibited incorporation of radioactivity into virions nor diminished the viral yield. Therefore, an influence of bafilomycin on assembly of infectious viral particles is rather unlikely. It should also be mentioned that the extent of label incorporated into progeny virions was found to substantially vary from experiment to experiment. We believe that this is due to the status of the cells which might influence the time of maximal viral synthesis and thus incorporation of radioactivity. To which extent HRV14 can be uncoated by ICAM-1 alone might also depend on the presence or absence (and the nature) of the pocket factor, presumably a fatty acid which is taken up at some stage during viral assembly and associates with a hydrophobic pocket in the viral capsid. The pocket factor is thought to be displaced upon receptor binding [22,23]. It is conceivable that the nature of this factor is subject to variations influencing the ease of uncoating. Quantification of the effects of bafilomycin on the yield of progeny HRV14 appears difficult; nevertheless, these data (and data presented earlier [8,9,14]) unequivocally demonstrate a clear difference between a minor group rhinovirus (HRV2) and a major group rhinovirus (HRV14) with respect to their sensitivity towards bafilomycin. Whereas uncoating of HRV2 does not take place upon elevation of the endosomal pH to neutrality, HRV14 can uncoat under these conditions although with variable efficiency.

Bafilomycin not only inhibits vesicle acidification but also budding of endosomal carrier vesicles from early endosomes [24]. As we have shown previously, HRV2 and fluid-phase marker en route to lysosomes are trapped in early compartments under these conditions [10]. Furthermore, we have also demonstrated that HRV14 gains access to the cytoplasm via endosome rupture [14] and that this process is not prevented by the drug. Thus it has to be assumed that uncoating of HRV14 can occur from early endosomes, does not require transfer of the virions to endosomal carrier vesicles or late endosomes and can be mediated by the receptor alone in the absence of endosome acidification.

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