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Entry of a heparan sulphate-binding HRV8 variant strictly depends on dynamin but not on clathrin, caveolin, and flotillin

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ABSTRACT

The major group human rhinovirus type 8 can enter cells via heparan sulphate. When internalized into ICAM-1 negative rhabdomyosarcoma (RD) cells, HRV8 accumulated in the cells but caused CPE only after 3 days when used at high MOI. Adaptation by three blind passages alternating between RD and HeLa cells resulted in variant HRV8v with decreased stability at acidic pH allowing for productive infection in the absence of ICAM-1. HRV8v produced CPE at 10 times lower MOI within 1 day. Confocal fluorescence microscopy colocalization and the use of pharmacological and dominant negative inhibitors revealed that viral uptake is clathrin, caveolin, and flotillin independent. However, it is blocked by dynasore, amiloride, and EIPA. Furthermore, HRV8v induced FITC-dextran uptake and colocalized with this fluid phase marker. Except for the complete inhibition by dynasore, the entry pathway of HRV8v via HS is similar to that of HRV14 in RD cells that overexpress ICAM-1.

Introduction

Human rhinoviruses are a major cause of the common cold (Heikkinen and Jarvinen, 2003). They include three species (HRV-A, B, and C) of the genus Enterovirus, family *Picornaviridae* (Palmenberg et al., 2009). Twelve HRV-A (the minor receptor group) (Vlasak et al., 2005b), bind low-density lipoprotein receptor (LDLR), LDLR related protein (LRP) (Hofer et al., 1994), and very-LDLR (VLDLR) (Marlovits et al., 1998), while the remaining 61 HRV-A and all 26 HRV-B utilize intercellular adhesion molecule 1 (ICAM-1) for cell entry and infection (Uncapher et al., 1991). The receptor(s) of the recently discovered HRV-C clade is not known (McErlean et al., 2008). The minor group virus HRV2 is internalized via the well-characterized clathrin-dependent endocytic pathway (Snyers et al., 2003); however, similar to other ligands, it can switch to different entry portals when this pathway is blocked (Bayer et al., 2001). Having once arrived in endosomal carrier vesicles or late endosomes, uncoating is triggered by the acidic pH (Konecsni et al., 2009; Neubauer et al., 1987; Prchla et al., 1994). Conversely, in major group HRVs the process is initiated by the receptor, ICAM-1, itself (Bayer et al., 1999; Greve et al., 1991) but assisted by the low endosomal pH (Nurani et al., 2003).

Attempts at adapting the major group HRV89 to bind LDL-receptors resulted in variants replicating in cells lacking ICAM-1 expression (Reischl et al., 2001; Vlasak et al., 2005a). HRV54 was even able to multiply in ICAM-1 negative rhabdomyosarcoma (RD) cells without prior adaptation. However, HRV54 wt as well as the above HRV89 variants had not acquired affinity for LDL-receptors but rather bound heparan sulphate (HS) and preserved their ability to attach to ICAM-1 (Khan et al., 2007).

HS is present ubiquitously on the surface of most animal cells and in the extracellular matrix where it has been implicated in numerous biological functions including cell–matrix and cell–cell adhesion, cell proliferation, motility and differentiation, blood coagulation, inflammation, tumor progression and invasion, tissue regeneration, lipoprotein metabolism and pathogen infections (reviewed in Bishop et al., 2007). Although many ligands and viruses attach to HS proteoglycans, data on cellular uptake are controversial. For example, entry of cationic peptides binding to HS was ascribed either to the clathrin-, the caveolin-, or the macropinocytic machinery (Duchardt et al., 2007; Jones, 2007; Poon and Gariepy, 2007). Eosinophil cationic

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protein, when attaching to HS on human bronchial epithelial Beas-2B cells exhibited characteristics of raft-dependent macropinocytosis (Fan et al., 2007). Finally, HS-binding cationic polyplexes and antibodies against HS were shown to enter in the absence of functional clathrin and caveolin but co-localized with flotillin and trafficked to late endosomes (Payne et al., 2007). An increasing number of viruses have also been shown to use HS as (an alternative) receptor but again, their entry pathway into the host cell is largely ignored (Spillmann, 2001). These ambiguous results indicate that uptake via HS is poorly understood. The involvement of proteoglycans in viral infection and drug delivery underscores the importance to investigate the mechanism underlying their uptake. We here report on entry and trafficking of HRV8v, a variant that binds HS and was selected for replication in ICAM-1 negative rhabdomyosarcoma (RD) cells. Employing pharmacological compounds, specific dominant-negative inhibitors, and immunofluorescence colocalization microscopy, we demonstrate that HRV8v enters RD cells via a dynamin-dependent pathway that is independent of clathrin, caveolin, and flotillin.

Results

Wild type HRV8 enters rhabdomyosarcoma cells but replicates inefficiently

HRV8 wt gave rise to CPE in ICAM-1 negative RD cells only upon challenge at >100 TCID50/cell after three days. However, upon continuous internalization for 30 min it readily entered RD cells and accumulated in vesicular structures seen by confocal immunofluorescence microscopy of a single confocal slice through the cell body (Fig. 1A and movie of deconvolved image stacks in Supplementary material). Previously, we obtained variants of HRV89 (HRV89M) upon 34 serial blind passages alternating between HeLa and Hep-2 cells that were able to replicate in these latter (ICAM-1 negative) cells (Reischl et al., 2001; Vlasak et al., 2005a). For HRV8, only three such passages in HeLa and RD cells were required for the appearance of a variant (HRV8v) that replicated and produced CPE equally in RD cells stably transfected to express human ICAM-1 (Newcombe et al., 2003) and in RD wild type cells. HRV8v was less stable at low pH than wt HRV8; at pH 5.6, a reduction of infectivity by almost 3 logs was observed (Fig. 1B). This pH value is attained in endosomal carrier vesicles and late endosomes in HeLa cells where it triggers uncoating of the minor group virus HRV2 (Brabec et al., 2003; Gruenberger et al., 1991). In the absence of the 'catalytic' activity of ICAM-1, HRV8v must be uncoated by the low pH. Thus, decreased viral stability in acidic environment correlates with productive infection in the ICAM-1 negative RD cells.

Employing the same strategy previously used to identify heparan sulphate (HS) proteoglycan as an alternative receptor for HRV54 (Khan et al., 2007) and the HRV89 variants mentioned above, it became clear that HRV8 also attaches to HS. This is exemplified by the inhibition of binding to RD cells with heparin (Fig. 2A), significantly reduced binding to CHO mutant cell lines pgsA-745 and pgsD-677 (Fig. 2B), and the complete inhibition of infection of RD cells by HRV8v in the presence of heparin (Fig. 2C). PgsA-745 is deficient in xylosyltransferase synthesis and unable to produce glycosaminoglycans whereas pgsD-677 is doubly deficient in N-acetylglucosaminyltransferase and glucuronyltransferase and fails to synthesize HS but produces threefold-higher levels of chondroitin sulphate than wt. As for the minor group virus HRV2 (Bayer et al., 1998) and the HS-adapted variants above, HRV8v infection via HS was completely blocked by bafilomycin A1, an inhibitor of endosomal H^+-ATPases. On the other hand, in RD-ICAM cells development of CPE was only slightly affected by the drug (not shown). Collectively, these data demonstrate that the more stable wt virus, although entering RD cells, only inefficiently uncoats without the aid of ICAM-1. Therefore, apart from receptor selection and entry, uncoating is a major determinant

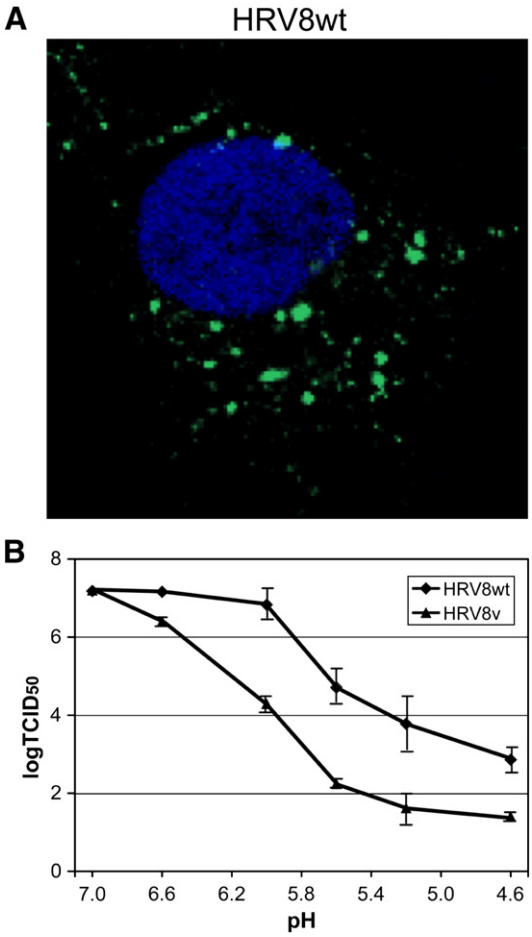

Fig. 1. HRV8 wt uncoating but not entry correlates with poor infection of ICAM-1 negative RD cells. A) RD cells were grown on glass cover slips and infected with HRV8 wt at 300 $TCID_{50}$/cell at 34 °C. After 30 min of continuous internalization, cells were washed and fixed with 4% paraformaldehyde. HRV8 was revealed with mouse antiserum followed by Alexa Fluor 488 secondary antibody. Nuclei were stained with DAPI. Cells were viewed under a LSM510 confocal microscope; one focal plane through the cell body is shown. B) Virus at 10^7 $TCID_{50}$ was incubated at 34 °C for 30 min in isotonic 100 mM MES buffer of the indicated pH values and, after re-neutralization, infectivity was determined by endpoint dilution. Mean and SD of 4 independent experiments.

for efficient infection. Note that all subsequent experiments were carried out with HRV8v to allow for functional assays (i.e. readout of entry/uncoating via cleavage of eIF4G and viral replication).

HRV8v shows different staining patterns when entering via ICAM-1 or via HS

HRV2 enters via clathrin-mediated endocytosis (CME) and HRV14 via a non-clathrin, non-caveolin, non-flotillin dependent route (Khan et al., 2010; Snyers et al., 2003). To study whether HRV8v follows any of these pathways, RD cells were co-infected with HRV8v and HRV2 for 30 min followed by fixation, specific staining, and immunofluorescence confocal microscopy. As seen in Fig. 3 (upper panels), there is only a marginal overlap (arrowheads) indicating that the two virus types localize to distinct compartments. HRV2 is delivered into early (EEA1-positive) endosomes and then transferred into late endosomes. Some of the HRV8v appears to attain also this early compartment. When the same experiment was conducted with HRV89M, another HS-binding variant (see above and Vlasak et al., 2005a), essentially complete overlap was observed (Fig. 3, middle panel). Co-localization was also evident upon internalization of HRV8v together with HRV14 in RD-ICAM cells, although the pattern