DISSERTATION

Titel der Dissertation

Receptor Specificity and Cellular Entry of Human Rhinoviruses

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"I know this is just not good enough but I dedicate this work to my family especially my mother whose prayers never left me alone not even for a single second"

Table of contents

Summary

Human rhinoviruses (HRVs), the major cause of the common cold, are characterized by more than 100 circulating types and are classified into two groups on the basis of receptor specificity. Minor group HRVs use members of the low-density lipoprotein receptor (LDLR) super-family while major group types bind to intercellular adhesion molecule-1 (ICAM-1). Analysing receptor discrimination at the atomic level, it has been shown that major group viruses contain ICAM-1 recognition signatures which are absent in the minor group HRVs. On the other hand, a single lysine residue is strictly conserved in the HI loop of VP1 of all minor group HRVs and is thought to play a key role in docking of the virus to LDL receptors. Interestingly, this lysine is also present in some ICAM-1 binding HRVs called K-type viruses. While characterizing the receptor specificity of the K-type HRVs, we found that HRV8 and HRV18 can interact with VLDLR concatemers. However, this interaction is much weaker when compared to HRV2 and they neither bind to LDLR immobilized on membranes nor on the cell surface. While screening these K-type HRVs for their ability to infect ICAM-1 negative rhabdomyosarcoma (RD) cells, we noticed that HRV54 is able to replicate in these cells. Infection inhibition assays indicated the involvement of heparan sulfate (HS) proteoglycans. By using receptor blocking assays, inhibition of sulfation, enzymatic digestion, and proteoglycan deficient cell lines, we show that wild type HRV54, without any adaptation, uses HS as an alternate receptor. However, infection via HS is less efficient than infection via ICAM-1 most probably due to inefficient virus entry and uncoating. HRV54 has a similar acid lability profile as HRV2 and in ICAM-1-expressing cells it can replicate in the presence of the H^+-ATPase inhibitor bafilomycin A1 whereas in ICAM-1 deficient cells its replication is completely blocked by the drug. Thus, using a non-catalytic receptor requires the virus to be highly sensitive to low pH. Moreover, the requirement for low pH sensitivity was also found in HRV8, another K-type virus that can bind and enter into RD cells via HS but fails to infect due to lack of uncoating. However, a variant (HRV8v) selected after few blind passages acquired the ability to replicate in these cells. It turned out that both viruses significantly differ in pH sensitivity, HRV8 wt being more stable than HRV8v. Collectively, these findings pinpoint a general trend in HRVs for binding to HS; this is intimately linked with becoming less stable to allow uncoating in the absence of catalytic receptor ICAM-1.

Where minor group HRVs depend on clathrin-mediated endocytosis, the pathway of major group HRVs and that of HS-binding viruses is largely unknown. We thus characterized the entry pathway(s) of these viruses when infecting the host cell via either of the three receptors. Immunofluorescence confocal microscopy demonstrated that the viruses were localized to different compartments within the infected cells depending on the type of receptor used for binding. Combining this technique with electron microscopy, dominant negative mutants and pharmacological inhibitor assays, we demonstrate that the major group virus HRV14 enters via dynamin-independent macropinocytosis into RD-ICAM cells where many viruses accumulate in long tubular structures. Entry of HRV8 into RD cells via HS exhibits similar characteristics with respect to co-localization with endocytic markers and pharmacological inhibitor profiles. However, HRV8 entry is dependent on functional dynamin and viruses accumulate in comparatively larger vesicular structures. The behaviour of HRV14 and HRV8, binding to quite different receptor molecules demonstrate the existence of both dynamin-dependent and dynamin-independent macropinocytosis in RD cells.

Zusammenfassung

Humane Rhinoviren (HRVs) stellen die Hauptursache für grippale Infekte dar. Zurzeit kursieren mehr als 100 Serotypen, welche aufgrund von Rezeptorspezifität in zwei Gruppen unterteilt werden. Minor group HRVs erkennen Vertreter der „low-density lipoprotein receptor" (LDLR) Familie, major group Rhinoviren binden an „intercellular adhesion molecule" 1 (ICAM-1). Die Analyse von Rezeptorunterscheidung auf atomarem Level zeigte, dass major group HRVs eine ICAM-1- Erkennungssignatur aufweisen, die in minor group HRVs nicht aufscheint. Bei minor group HRVs ist ein Lysin streng konserviert, es kommt im HI loop vonVP1 von allen Vertretern der minor group vor. Mit hoher Wahrscheinlichkeit nimmt es eine Schlüsselrolle bei der Erkennung von LDL- Rezeptoren ein. Interessanterweise kommt dieses Lysin auch bei HRVs vor, die an ICAM-1 binden; diese werden K-Typ Viren genannt. Während der Charakterisierung der Rezeptorspezifität von K-Typ Viren fanden wir heraus, dass HRV8 und HRV18 mit VLDLR- Konkatemeren interagieren können. Im Vergleich zu HRV2 ist diese Interaktion allerdings viel schwächer, des Weiteren sind K-Typ Viren nicht in der Lage an membranständige oder von Zellen präsentierte LDL- Rezeptoren zu binden. Während der Überprüfung ob K-Typ Viren ICAM-1 defiziente Rhabdomyosarcomazellen (RD) infizieren können, fiel auf, dass HRV54 in der Lage ist in diesen Zellen zu replizieren. Infection inhibition assays gaben einen Hinweis darauf, dass Heparansulfat-(HS) in die Zellerkennung durch HRV54 involviert sein könnte. Mithilfe von Rezeptorblockade, Inhibierung der Sulfatierung, enzymatischem Verdau und Verwendung von HS- defizienten Zelllinien zeigen wir, dass HRV54 Wildtyp (wt), ohne jegliche Adaptierung, HS als alternativen Rezeptor nutzen kann. Infektion via HS ist allerdings im Vergleich zu Infektion via ICAM-1 weniger effizient, vermutlich aufgrund von schlechterer Virusaufnahme in die Zelle und Freisetzung des viralen Genoms. HRV54 ähnelt HRV2 bezüglich Säurelabilität. In ICAM-1- exprimierenden Zellen kann HRV54 auch in Gegenwart des H^+-ATPase- Inhibitors Bafilomycin A1 replizieren. In Zellen, die ICAM-1 nicht exprimieren wird die Replikation von HRV54 durch Bafilomycin komplett blockiert. Folglich nötigt der Gebrauch eines nicht katalytisch wirkenden Rezeptors das Virus zu erhöhter Säurelabilität.

Dieser Bedarf an erhöhter Säurelabilität wurde auch bei HRV8, einem weiteren K-Typ Virus Vertreter festgestellt. HRV8 kann ebenfalls mittels HS an RD Zellen binden und aufgenommen werden, löst allerdings keine Infektion aus da das virale Genom nicht freigesetzt wird.

Nach einigen Serienpassagen/ Blindpassagen wurde eine Variante (HRV8v) selektiert, die die Fähigkeit in RD Zellen zu replizieren gewonnen hatte. HRV8 und HRV8v unterscheiden sich stark bezüglich pH- Sensitivität, wobei HRV8 wt stabiler ist als HRV8v. Zusammengefasst weisen diese Beobachtungen darauf hin, dass ein allgemeiner Trend bei HRVs besteht an HS zu binden. Die Neigung an HS zu binden ist eng mit geringerer Säurestabilität verbunden, um eine Konformationsänderung des Capsids auch ohne die Hilfe des diesbezüglich katalytisch wirksamen Rezeptors ICAM-1 zu gewährleisten.

Minor group HRVs werden durch Clathrin- abhängige Endozytose in die Zelle aufgenommen, für major group HRVs, und HRV- Serotypen, die an HS binden, ist der Mechanismus, durch den sie in die Zelle aufgenommen werden weitgehend unbekannt. Wir charakterisierten daher die Aufnahmemechanismen dieser Viren und der drei korrespondierenden Rezeptortypen. Mittels konvokaler Immunofluoreszenzmikroskopie konnte gezeigt werden, das Viren, je nachdem welcher Rezeptor benutzt wurde, in unterschiedliche Zellkompartimente transportiert wurden. Durch Kombination dieser Technik mit Elektronenmikroskopie, der Herstellung von dominant- negativen Mutanten, und der Austestung von pharmakologischen Inhibitoren konnten wir zeigen, dass HRV14, als Repräsentant der major group HRVs, mittels Dynamin- unabhängiger Makropinozytose in RD-ICAM Zellen aufgenommen wird und sich an tubulären Zellmembraninvaginationen ansammelt. Aufnahme von HRV8 in RD Zellen, vermittelt durch HS zeigt ähnliche Charakteristika bezüglich der Kolokalisation mit Endozytosemarkern und den inhibitorischen Effekten von pharmakologisch aktiven Substanzen. Die Aufnahme HRV8 ist aber im Gegensatz zum Zelleintritt von HRV14 abhängig von funktionellem Dynamin, des Weiteren wird HRV8 in vergleichsweise größeren vesikulären Strukturen konzentriert. Das unterschiedliche Rezeptorbindungsverhalten und die verschiedenen Invaginationsstrukturen bei der Aufnahme von HRV14 und HRV8 demonstrieren die Existenz von sowohl Dynamin- abhängiger als auch – unabhängiger Makropinozytose in RD Zellen.

1. Introduction

Picornaviruses

Picornaviruses are small single stranded RNA viruses with positive polarity within the virus family *Picornaviridae* which is characterized by more than 200 discrete serotypes and several picorna-like viruses. *Picornaviridae* is one of the heterogeneous and ancient virus families and has considerable impact on economy and human health due to some prominent members like polio, foot and mouth disease virus (FMDV), rhino and coxsackie viruses. Poliomyelitis is one of the oldest humanity problem traced back in Egyptian hieroglyphs more than 4000 years ago and still causing the death of unvaccinated subjects in underdeveloped countries. FMDV is another vital pathogen of live stock and an outbreak of this virus in the late 20th century in Great Britain led to the culling of approximately 25 million cattle, devastating the British cattle industry. Finally, rhinoviruses are the major cause of all mild infections of the upper respiratory tract and occasionally more fatal lower respiratory tract with an estimated economical impact of 5 billion USD per year in the USA alone and huge loss of working days from schools and industries.

Classification

Conventionally, picornaviruses have been classified, identified, and differentiated on the basis of physical and antigenic properties, like acid stability and virion density, and by neutralization with specific antisera. For example enteroviruses, cardioviruses, and hepatitis A virus are acid stable, supporting pH ≤3, whereas the rhinoviruses and aphthoviruses are acid labile and are readily inactivated at pH ≤6. This difference is probably the reason for their pathogenecity as the acid labile rhinoviruses are frequently restricted to only nasopharyngeal and occasionally to the lungs epithelium with no apparent need for acid stability. On the other hand, enteroviruses primarily infect cells of the intestinal epithelium and lymphoid cells of the gut and have to pass the low pH in the stomach (Newman et al., 1973). Picornaviruses also differ widely in virion density. The sedimentation coefficient of native poliovirus is 156S while the sedimentation coefficient of rhinovirus is 149S (Fields et al., 1996). Apart from physical properties, more recently molecular techniques such as nucleotide sequencing, has

been applied to picornavirus identification and classification which has resulted in the establishment of various new genera and rearrangements of the already known ones.

In contrast to the previous classification into nine genera, the family *picornaviridae* has been squeezed into 8 genera after merging two rhinovirus species into enteroviruses and the genus rhinovirus is not any more in existence (table 1.1).

Genus	Species
Enterovirus	Human *enterovirus A*, *Human enterovirus B*, *Human enterovirus C*, *Human enterovirus D*, *Simian enterovirus A*, *Bovine enterovirus*, *Porcine enterovirus A*, *Porcine enterovirus B*, *Human rhinovirus A* and *Human rhinovirus B*
Cardiovirus	*Encephalomyocarditis virus* and *Theilovirus*
Aphthovirus	*Foot-and-mouth disease virus* and *Equine rhinitis A virus*.
Hepatovirus	Hepatitis virus A and Avian encephalomyelitis virus (proposed name for Avian encephalomyelitis-like viruses)
Parechovirus	*Human parechovirus* and *Ljungan virus*
Erbovirus	*Equine rhinitis B virus*
Kobuvirus	*Aichi virus* and *Bovine kobuvirus* (recently described in pigs; Porcine kobuvirus)
Teschovirus	*Porcine teschovirus*

Table 1.1: Current classification scheme of family *Picornaviridae*. Note genus rhinovirus is merged in enterovirus and there are now total 8 genera instead of nine (**http://www.picornaviridae.com**).

The complete genome of three other picornaviruses has been recently sequenced and they have been proposed as belonging to novel genera. These viruses are duck hepatitis virus 1, human cosavirus and seal picornavirus 1. Proposals to designate them as such are in preparation and thus in the near future, the family picornaviridae will probably consist of 14 genera and 33 species (www.picornaviridae.com).

Human Rhinoviruses

Human rhinoviruses (HRVs) are characterized by 102 circulating types and are a major etiologic cause of common cold. These types have been differently classified, following genetic clustering and type of the receptor used for binding to the host cells. Based on capsid protein sequences, they are divided into two phylogenetic species called HRV-A and HRV-B. Twenty five serotypes are clustered within the group A while the rest into group B (table 1.2). More recently, new HRV strains have been discovered by using molecular techniques instead of conventional culture-based diagnostics (Arden et al., 2006; Kistler et al., 2007; Lamson et al., 2006; Lau et al., 2007; Lee et al., 2007; McErlean et al., 2007). Sequence analysis of these newly identified strains revealed the existence of new HRV-A strains and certain other that might represent an additional HRV genus termed HRV-C (Lau et al., 2007; Lee et al., 2007). Notably these strains do not grow in tissue culture and most probably went unnoticed due to this fact (Palmenberg et al., 2009).

1A									H
1B	11	21	31	41	51	61	71	81	*91*
2	12	22	32	*42*	*52*	62	*72*	82	*92*
3	13	23	33	43	53	63	73	*83*	*93*
4	*14*	24	34	44	54	64	74	*84*	94
5	15	25	*35*	45	55	65	75	85	95
6	16	*26*	36	46	56	66	76	*86*	96
7	*17*	*27*	*37*	47	57	67	77	87	*97*
8	18	28	38	*48*	58	68	78	88	98
9	19	29	39	49	59	*69*	*79*	89	99
10	20	30	40	50	60	*70*	80	90	100

Table 1.2: Phylogenetic division of HRVs. HRV-A are shown in bold while HRV-B in regular letters. Minor group rhinoviruses have been shown in red whereas major group HRVs in black color. Note, two types 23 and 25 shown in orange color originally classified as major group HRVs have been placed in minor group HRVs as they use LDLR for binding and infection. Type 87 shown in blue is more related to enteroviruses.

According to the receptor specificity, HRVs are classified as major and minor receptor group (Abraham and Colonno, 1984; Colonno et al., 1986; Lonberg-Holm and Korant, 1972; Uncapher et al., 1991). Using binding compettition and cell protection assays with a monoclonal antibody against human intercellular adhesion molecule-1 (ICAM-1), 91 serotypes were grouped as major receptor group HRVs while the remaining 10 serotypes competed for a different protein and were termed as minor group HRVs (Greve et al., 1989; Staunton et al., 1989b; Tomassini and Colonno, 1986). This receptor protein was identified as low density lipoprotein receptor (LDLR) by Hofer et al (Hofer et al., 1994b). Further work in this direction revealed that not only LDLR but also other members of this superfamily like very low density lipoprotein receptor (VLDLR) and LDLR like protein (LRP) can also participate in the attachment of the minor group HRVs to their host cells (Marlovits et al., 1998a; Marlovits et al., 1998b; Marlovits et al., 1998c). Interestingly, all but not one serotype (HRV87) could be classified by both of these criterions as this virus was shown to bind to a sialidase-sensitive glycoprotein and could not segregate in either of the genetic cluster (Uncapher et al., 1991). Subsequent investigation showed that HRV87 is antigenically and genetically more identical to enterovirus 68 (EV68) and is related to EV70, the other member of human enterovirus group D although the acid stability profile of both viruses is different (Savolainen et al., 2002). Two other serotypes, HRV23 and HRV25 which were originally designated as major receptor group viruses, in fact turned out to interact with minor group receptors and were rearranged and placed into minor group HRVs (Vlasak et al., 2005b). Moreover, HRV8 and HRV95 appeared to be the same serotype and same is true for HRV-Hanks (H) and HRV21. Taking all these changes into account, the number of HRV types within the two species reduced from 102 to 99 except newly discovered strains.

Interestingly, some major group HRVs (shown in red colour) possess double receptor specificity and can use heparan sulfate proteoglycan for productive infection in addition to their prime receptor ICAM-1 (Khan et al., 2007).

Human Rhinovirus types		
Minor receptor group HRVs	**Major receptor group HRVs**	
1A, 1B, 2, 23, 25, 29, 30, 31, 44, 47, 49, 62	3, 4, 5, 6, 7, **8**, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 24, 26, 27, 28, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 45, 46, 48, 50, 51, 52, 53, **54**, 55, 56, 57, 58, 59, 60, 61, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100	

Table 1.3: Classification of HRVs on the basis of receptor specificity. 12 HRV types out of 99 bind to members of low density lipoprotein superfamily and are named as minor group HRVs whereas the rest types use intercellular adhesion molecule-1 and are called major group HRVs. Note the viruses shown in red (HRV8 and HRV54) possess double receptor specificity and can bind to HS as well.

HRV Virions

The capsid of picornaviruses is approximately 30 nm in diameter and is composed of four structural proteins, VP1, VP2, VP3, and VP4. These proteins are arranged in 12 pentameric subunits and each subunit is composed of protomers with one copy of each of the four viral proteins. The mature capsid has pseudo-threefold axis T=3 icosahedral symmetry. VP1, VP2, and VP3 are arranged on the surface of the virion and thus comprise not only the major immunogenic sites (with VP1 being immuno-dominant) but also play a major role in interactions with host receptors. VP4 is comparatively small, lies inside the shell and forms an interface between the capsid and the internal RNA genome in conjunction with the amino termini of VP1 (about 70 residues in HRVs) and VP2. These residues are generally disordered in the immature capsid as VP0 is cleaved into VP2 and VP4 during the maturation process. The N-terminus of VP4 is myristoylated and is thought to be required in pentameric assembly and might also play an important role in release of virion genome (Ansardi et al., 1992; Chow et al., 1987; Moscufo and Chow, 1992). Capsids of entero and rhinoviruses contain a peculiar groove around the five-fold axis called canyon. It is formed at the junction of VP1 (north rim) and VP2 and VP3 (south rim) while the floor of the canyon is mainly composed of the GH loop of VP1. Although the canyon was proposed to be the site of receptor binding, many natural escape mutants had mutations within the hyper-variable regions located in four

distinct antigenic patches on the viral surface on either rim of the canyon (Rossmann et al., 1985; Sherry et al., 1985; Sherry and Rueckert, 1985). According to the canyon hypothesis, antibody being more bulky would not have access to the canyon floor due to narrow rims while receptor being slim could easily reach to the floor of the canyon (Rossmann, 1985). The canyon was indeed proved to be the receptor binding region by cryo-electron microscopy of HRV16, HRV14 and polio virus with their respective soluble ICAM-1 and polio receptors. The receptor was also predicted to have a role in viral stability and its disassembly during uncoating. However, the canyon hypothesis was challenged by the finding that an antibody could penetrate into the canyon equally well to the receptor (Smith et al., 1996). Contrary to polio and major group HRVs, binding of minor group HRVs has been spotted on the star shaped dome at the five-fold axis of the icosahedral capsid (Hewat et al., 2000).

The role of the canyon in virus stability was further strengthened by the discovery of the pocket factor, a fatty acid in a hydrophobic pocket underneath the canyon floor. WIN compounds that have been used as antivirals, in fact replace the pocket factor and stabilize the particle either directly or via blocking the access of ICAM-1 (Gruenberger et al., 1991; Pevear et al., 1989; Smith et al., 1986). Thus, differences in virion stability between rhinovirus serotypes might also reflect the amount of pocket factors present in the respective virus.
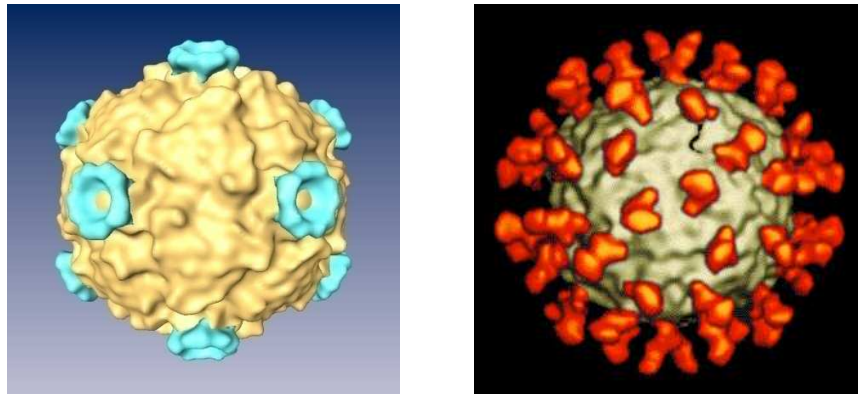
Figure 1.1: Interaction of the rhinovirus receptors to the virion. Binding of VLDLR (repeat 3) to HRV2 (left) shows the receptor density around the five-fold axis whereas ICAM-1 penetrates within the canyon, the crevice around the five-fold axis (right).

HRV Genome

The genome of rhinoviruses is made up of a single stranded RNA of 7.1 kb length. The genome can be divided into four distinct parts, the 5' untranslated region (5'UTR), open reading frame (ORF), 3' non-coding region (3'NOR) and poly A tail. The viral protein VPg is linked to the 5' pUpUp region through a phosphodiester linkage to the hydroxyl group of its tyrosine residue (Wimmer, 1982). VPg plays a very important role in priming viral RNA synthesis and might be involved in its penetration through cellular membranes during genome release. Unlike to the majority of the cellular messenger RNAs, picornaviruses lack the m^7G cap, but instead possess a conserved region of extensive secondary structure serving as an internal ribosomal entry site (IRES). The IRES mediates the formation of a pre-initiation template/ribosome complex securing proper translation when CAP-dependent translation is blocked during rhinovirus infection (Agol, 2002; Kozak, 1989).

The ORF can be divided into three main regions from P1-P3 and is translated in a single polyprotein. The P1 region codes for structural proteins giving rise to VP4, VP3, VP2 and VP1.The P2 and P3 regions encode non-structural proteins which are involved in co-translational cleavage of the polyprotein (viral proteases 2A and 3C) (Palmenberg et al., 1979; Toyoda et al., 1986), host cell shut off (proteases 2A, 3C, and L) (Bernstein et al., 1985), host range determination (2B, 2C) (Lomax and Yin, 1989; Yin and Lomax, 1983) and replication of the viral RNA (2C, 3AB, VPg and RNA-polymerase 3D) (Flanegan et al., 1977; Li and Baltimore, 1988; Nomoto et al., 1977; Pallansch et al., 1980; Semler et al., 1981; van Dyke et al., 1982).
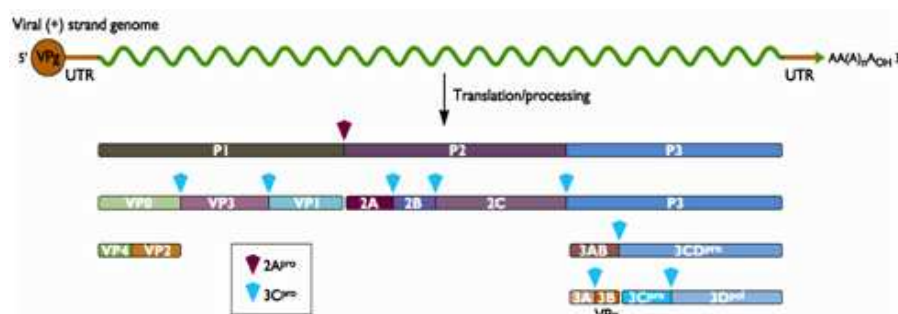
Figure 1.2: Genome organization of picornaviruses. Schematic representation of RNA with three distinct parts has been shown. RNA is translated into a polyprotein with P1, P2 and P3 region which upon autocatalytic cleavage give rise to 12 structural and functional proteins along with at least 3 major intermediates.

Viral Replication Cycle

Being obligate intracellular parasites, viruses must get into the host cells to start their multiplication. Picornaviruses are non-enveloped viruses and cannot directly fuse with the cell membrane and thus require their doorman for entry into the target cells to cross this barrier. Therefore the host tropism for a virus is limited by the presence of particular receptor molecules on the surface of target cells and variation of the receptors among different species is a major determinant of the host range. In general the life cycle of rhinoviruses can be divided into the following events.

1: Receptor recognition and binding, 2: Entry, 3: Uncoating, 4: Replication and translation, 5: Virus assembly and release.
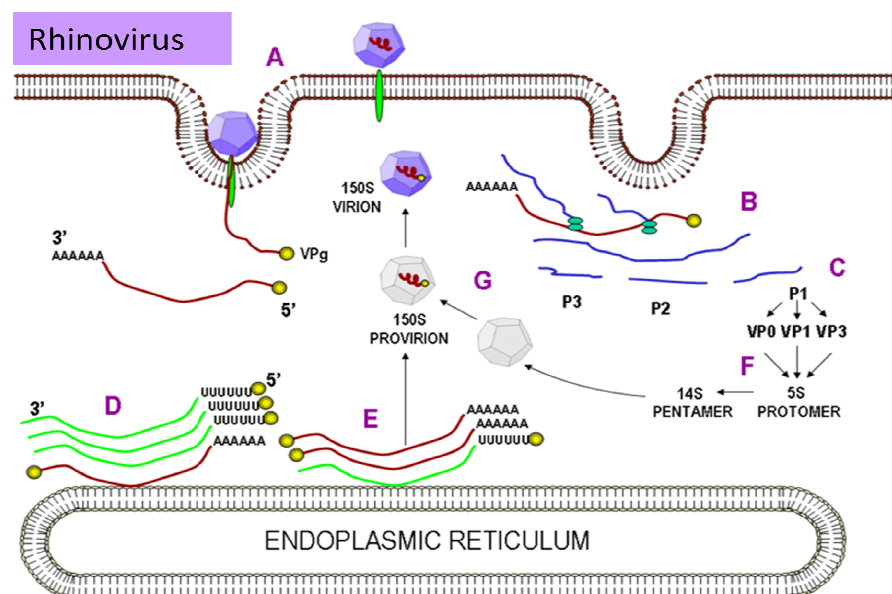
Figure 1.3: Virus life cycle. A) Virus binding to the receptor and entry which results in the release of RNA. B) Direct tranlation of incoming RNA and production of polyprotein. C) Self-cleavage of polyprotein into precursor structural and functional proteins. D) Production of –ve strand RNA for the template of genome multiplication. E) Synthesis of +ve strand RNA for encapsidation. F) Assembly of capsid proteins. G) Encapsidation of RNA to produce provirions and subsequent maturation into infectious virion.

Receptors

The most astonishing result of studies focused on the initial events of animal virus infections is the diversity of cell surface proteins serving as receptors. There is no obvious reason why one virus species would use receptor A while its very close relative may have selected a totally different molecule B. For example major group HRVs use ICAM-1 while minor group viruses utilize an entirely different class of receptors called LDLR superfamily. On the other hand two entirely different viruses, such as small RNA coxsackievirus B and the large DNA adenovirus type 2, use the same receptor called coxsackie-adenovirus receptor (CAR). Although retroviruses possess the most diverse collection of receptors, picornaviruses too have a great ingenuity in recognizing the receptors on the surface of target cells. Picornaviruses use eight main receptors along with many related or co-receptors.

Major group HRVs recognize ICAM-1 while minor group HRVs make use of LDLR, VLDLR and LRP to obtain access to the host cells. At the same time, some major group HRVs have shown to exploit cell surface heparan sulfate proteoglycan in addition to ICAM-1 especially in ICAM-1 negative cells (Khan et al., 2007). The basis of receptor discrimination is still a major open question and the specificity of rhinovirus receptor selection is not entirely clear.

Members of the low-density lipoprotein receptor family; receptors of minor group HRVs

A protein that showed binding activity for a minor group type HRV2 was first isolated from the supernatant of HeLa cell culture and was identified as the human lipoprotein receptor (Hofer et al., 1992; Hofer et al., 1994a). Continued work in understanding of the mechanism of interactions of minor group viruses with their receptors resulted in the discovery of other members of this family that could also interact with HRV2 (Ronacher et al., 2000). These include the very low density lipoprotein receptor (VLDLR) and LDLR related protein (LRP). Thus minor group HRVs could be neutralized by more than one protein belonging to the LDLR family.

The LDL receptor family is a class of transmembrane proteins. In addition to their specialized functions as mediators of cellular lipid uptake, they have over the last few years, also been recognized for often unrelated roles as cellular signal transducers or signal modulators. These cell surface receptors fall into two major groups: endocytic receptors that bind their cargo in the form of lipid-carrying lipoproteins, mediate their internalization and eventually lysosomal degradation, and a second group which promotes lipid exchange at the plasma membrane without uptake of protein component of the particle. Due to this functional diversity, the members of the LDL receptor family are ubiquitously expressed and highly conserved throughout different species. The genes coding for this family consist of structurally closely related seven members. These members include LDL receptor (Yamamoto et al., 1984), VLDL receptor (Takahashi et al., 1992), the LDL related protein (LRP) (Herz et al., 1988), megalin (Saito et al., 1994), ApoE receptor-2 (ApoER2) (Kim et al., 1996; Novak et al., 1996), MEGF7 (Nakayama et al., 1998) and LRP1b (Liu et al., 2000).

Each member is characterized by the presence of five distinct domains or sequence motifs. The extracellular region consists of one to several clusters of varying numbers of cysteine-rich complement-type A repeats called the ligand binding domains, epidermal growth factor repeats and an additional highly *O*-glycosylated threonine and serine rich region located at the carboxy-terminus. This is unique only for the LDLR, the VLDLR and ApoER2. The receptors are anchored in the plasma membrane by a single transmembrane domain that is followed by a short cytoplasmic tail. The intracellular regions of the different receptors share little sequence similarity except for the presence of at least one copy of a NPxY motif that serves as an internalization signal to steer the loaded receptors into clathrin-coated pits (Chen et al., 1990). The arrangements of the 40 amino acid ligand binding modules that show slight sequence variations among themselves, enable the members of the LDLR family to recognize a large number of structurally and functionally unrelated ligands (Nykjaer and Willnow, 2002). Furthermore, several members of this family associate with co-receptors serving as binding sites for metabolites which help to extend their specificity profile for the ligands.

Mutations within each domain strongly interfere or abrogate altogether the normal functions of the whole receptor. For example mutations in the ligand binding domain abolish the interaction with their ligands and hence receptors are not functional any more. Mutations in the EGF precursor homology domain, which is involved in the pH induced ligand release and

subsequent recycling of the receptor, strongly affect the receptor recycling and hence cause the degradation of the receptor along with the ligands. Finally, in case of substitution or deletion in the cytoplasmic tail, the receptor binds the ligands but can not internalize or is cleaved off the cell surface respectively (Hobbs et al., 1992).
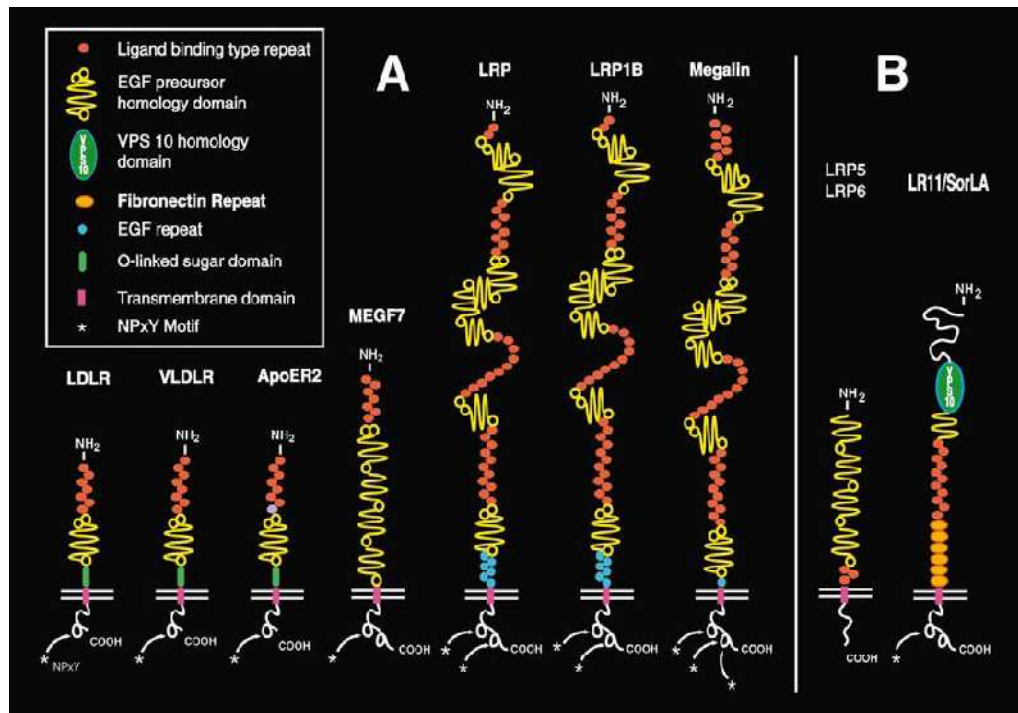
Figure 1.4: The LDL receptor superfamily. A) The core of the LDL receptor gene family consists of the LDL receptor (LDLR), VLDL receptor (VLDLR), apolipoprotein E receptor-2 (apoER2), multiple epidermal growth factor containing protein 7 (MEGF7), LDL receptor-related protein (LRP), LRP1b, and megalin.
B) Distantly related receptors. These comprise the Wnt receptor LRP6, the closely related LRP5, and LR11/SorLA. Their domain structure is different from that of the core members of the family. Note LRP5 and LRP6 do not contain NPxY motifs in their cytoplasmic tails.

The LDL receptor is one of the best studied members of this family (Goldstein et al., 2001). The receptor is well known for its role in plasma cholesterol homeostasis and removal of LDL from the circulation. Functional deficiencies of LDLR result in familial hypercholesterolemia (FH) in humans (Hobbs et al., 1992; Ishibashi et al., 1993; Tolleshaug et al., 1983), which is characterized by elevated plasma lipoprotein and cholesterol levels and causes

artherosclerosis and coronary artery disease. LDLR contains 7 ligand binding modules which are structurally independent of each other (Bieri et al., 1998; North and Blacklow, 1999). Each domain is flanked by short flexible linkers that provide considerable freedom of rotation and allow the modules to rearrange themselves and to bind to the variety of ligands (Kurniawan et al., 2000). Entry of the ligands is essentially via clathrin-mediated endocytosis as the tetra amino acid (NPxY) sequence is highly conserved in all receptors and triggers the clustering of receptors in the coated-pits (Chen et al., 1990; Davis et al., 1987).

VLDLR and ApoER2 have almost identical primary structure and size to that of LDLR and were identified through genome wide searches for LDL receptor related genes (Kim et al., 1994b; Novak et al., 1996; Takahashi et al., 1992). Due to this striking resemblance and binding of both receptors to ApoE *in vitro*, lipoprotein metabolism was proposed as their major function. However, neither protein is expressed in the liver. For example the primary sites of VLDLR expression are endothelium and muscles (Jokinen et al., 1994; Wyne et al., 1996) whereas expression of ApoER2 is restricted to neural tissue and testes of adult mice (Gotthardt et al., 2000; Stockinger et al., 1998). VLDLR has 8 ligand binding repeats while ApoER2 contains 7. Mutations of VLDLR have a significant effect on brain development rather than plasma LDL level disorders.

LRP is one of the largest cell surface proteins and was identified as a second member of the LDL receptor family (Herz et al., 1988). It contains 31 ligand binding repeats which are interrupted by EGF precursor homology domains. LRP binds to ApoE-containing lipoprotein but not LDL, thus LRP cannot compensate for the functions of LDLR whereas the latter can fully replace the normal functions of LRP. LRP is a multifunctional protein and interacts with variety of secreted as well as resident cell surface proteins.

Interactions of the minor group HRVs with their receptors

The basis of minor group HRV receptor specificity is still largely at vague. Where major group HRVs showed binding within the canyon, binding of the minor group HRVs to their LDL receptors through site-directed mutagenesis was found to be outside this cavity (Duechler et al., 1993). As VP1 is the major protein of the capsid, the complete sequence of this protein for all HRV types was used to understand the basics of virus receptor interactions. Comparison of VP1 sequence revealed only a lysine strictly conserved within HI loop of all

minor group HRVs. However, the other residues in this binding region are highly divergent. At least some minor group HRVs also have the K, a T and an E but a peptide containing this TEK motif was tested for competition and no inhibition was found. The first structural view was provided when soluble VLDLR fragments complexed to HRV2 were analyzed via cryo-electron microscopy. Although the image obtained was of low resolution (15 Å) it showed a clear density around the fivefold axis of the icosahedral symmetry (Hewat et al., 2000). Furthermore, the strictly conserved lysine in the exposed HI loop of VP1 was shown to play a key role in establishing the receptor-virus interactions. Interestingly, 9 major group HRVs also contain this lysine essentially at the same position. These major group HRVs are called K-type viruses. When these K-type viruses were tested for their ability of binding to the minor group receptors, it was found that infection by these viruses was inhibited in the presence of ICAM-1 binding antibody (R6.5) (Vlasak et al., 2003). Moreover, they could not use minor group receptors for infection of ICAM-1 negative (RD) cells although some of them showed a weak binding to the VLDLR concatemers on virus overlay blot assays. However, this interaction was much weaker when compared to HRV2 (present study). On the other hand replacing the K in HRV2 resulted in the loss of infectivity most likely due to loss of interaction with the receptor. This evidence strongly suggested that the lysine of the HI loop is a key residue for minor group HRVs receptor docking but is not sufficient alone for significant interaction. Interestingly, phylogenetic analysis of minor group and K-type major group HRVs revealed that the former were more related to the latter group than with each other. Generation of 3-D models of VP1 of various viruses and subsequent energy calculations pointed out the overall positive charge density at the receptor binding region in minor group HRVs which is absent in majority of K-type viruses. However, there are few major group HRVs with comparable positive charge on this region but they lack the lysine. All these calculations and predictions led to the formulation of the belief that overall positive charge and conserved lysine are the key players for minor group virus-receptor interactions. Whereas, the presence of a positive charge is well in accord to the highly conserved acidic residues present in the ligand binding domains of all minor group receptors (LDLR, VLDLR and LRP), the importance of the lysine is still a major stake to resolve the enigma of the minor group virus-receptor interaction. To this end, receptor specificity of recently isolated variants

which are very close to the minor group HRVs and carry an arginine (R) instead of lysine (K) will be interesting to study.
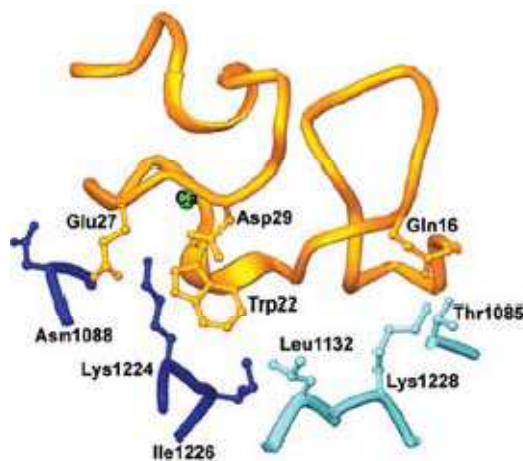
Figure 1.5: Interaction of HRV2 with V3 at atomic levels as determined by X-ray crystallography. Ribbon shaped representation of HRV2-V3 where receptor has been shown in yellow and virus protein VP1 in blue and light blue. Interaction between receptor and virus include ionic (Lys1224-Trp22), hydrophobic (Ile 1226&Leu1132-Trp22) and hydrophilic (Lys1228&Thr1085-Gln16) (Verdaguer et al., 2004).

Intercellular adhesion molecule-1 (ICAM-1); the receptor of major group HRVs

Intercellular adhesion molecule-1 (ICAM-1, CD54) is a transmembrane glycoprotein whose presence on the surface of human cells makes them vulnerable to infections by majority (87) of HRV types. ICAM-1 belongs to immunoglobulin (Ig) superfamily and is composed of three major parts such as an extracellular region comprising of 5 Ig like domains (D1-D5), a transmembrane region and a short cytoplasmic tail (figure 1.6). Each Ig domain can be characterized by a typical building block of antibodies where its structure is composed of two tightly packed antiparallel β-sheets against each other. Various domains are connected through several loops following a well-defined topology and linked by one or two disulfide bridges. Other members of this family are ICAM-2 to ICAM-5 that share basic structure and organization. All ICAMs show a strong similarity in sequence and organization of domains, structure and function. However, they also differ in the number of Ig domains and cell type specific distribution and level of expression (Bella and Rossmann, 2000). Furthermore all

these proteins are highly glycosylated but there is no common pattern of glycosylation among the various types.
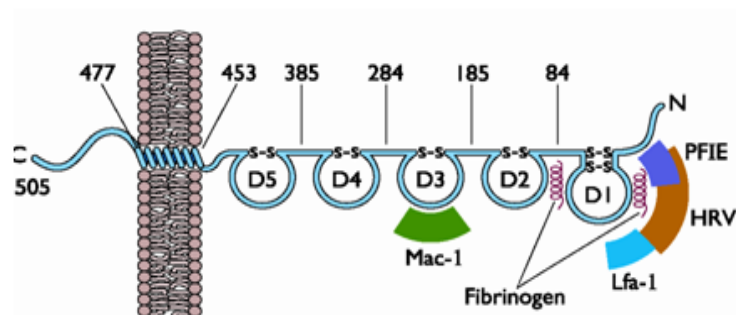
Figure 1.6: Structure and composition of ICAM-1. Diagram shows sites of glycosylation (lollipop-shaped structures) and the approximate location of binding sites of LFA-1, Mac-1, human rhinoviruses, fibrinogen, and *Plasmodium falciparum*-infected erythrocytes (PFIE) (Bella et al., 1999).

ICAM-1 is expressed on the surface of many cells involved in immune and inflammatory response. Normally its concentration is low but cytokines can enhance expression as required for the execution of the immune response. ICAM-1 triggers adhesion of the leukocytes to the epithelial cells which results in the accumulation of these cells at the site of infection and hence causes inflammation. Adhesion of leukocytes with ICAM-1 expressing epithelial cells is mediated by the binding of ICAM-1 to its natural ligands that include leukocyte function-associated antigen-1 (LFA-1, CD11a/CD18) and macrophage-1 antigen (Mac-1, CD11b/CD18), both belonging to the integrin family of membrane proteins.

Interactions of major group HRVs with their receptor

The interaction of the major group HRVs with their receptor ICAM-1 has been comparatively well characterized. According to the canyon hypothesis it was suggested that the receptor would bind in the crevice that surrounds each of the fivefold vertices. Site directed mutagenesis approaches revealed that mutations in the canyon floor dramatically changed the interactions between virus and ICAM-1. Furthermore, later on it was shown that a series of

drugs could enter a hydrophobic pocket located under the canyon floor through the pore at the base of the canyon. These drugs interfered with virus uncoating and/or attachment of the receptor and suggested that mutation in the canyon indeed influenced the interaction with the receptor. Binding of ICAM-1 in the canyon was confirmed by studies employing cryo-EM of complexes between HRV16 and two N-terminal domains (D1 and D2) of ICAM-1. Sequence analysis and mutational assays revealed that the interaction appeared to have an important electrostatic character, since the interacting surface of the tip of D1 and the canyon shows a striking complementarity of the charge distribution (Bella and Rossmann, 1999).
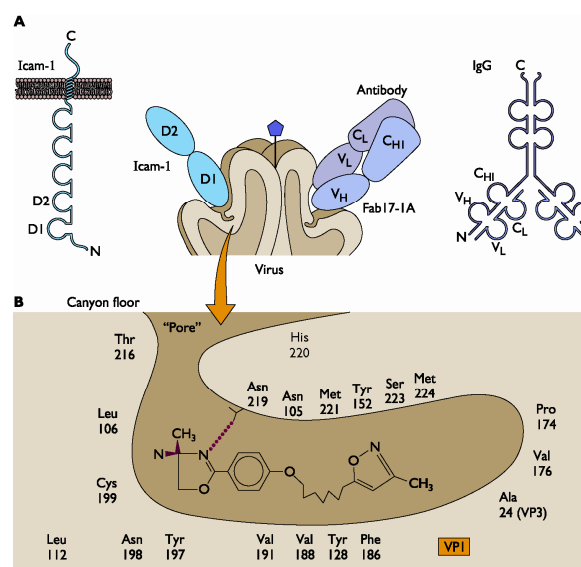
Figure 1.7: Binding of ICAM-1 in capsid canyon. A) Two N-terminal domains (D1 and D2) of ICAM-1 interact with HRV capsid and bind within the canyon. Fab antibody can also penetrate into the cavity and inhibits the attachment of ICAM-1. B) According to the canyon hypothesis the pocket factor is present at the floor of canyon which provides stability to the virion. This pocket factor is expelled out by binding of the receptor.

Heparan sulfate proteoglycans (HSPGs)

An ever increasing number of bacteria and viruses have been shown to use cell surface HSPGs as a receptor or co-receptor. The list has accumulated more than 20 virus members within the last few years and table 1.3 demonstrates the diversity with representatives from

human and animal, enveloped and non-enveloped, and DNA and RNA viruses. HS is a highly sulfated polysaccharide carrying a considerable number of negative charges under physiological conditions. These negatively charged groups are therefore ideal candidates for viral proteins carrying positive surface charge which provide initial interactions between virus and the target cells. However, the interaction with viruses might be far more complex than just ionic. For instance, recent studies have shown that human immunodeficiency virus 1 (HIV-1), dengue virus and herpes simplex virus 1 (HSV-1) recognize a specific HS saccharide sequence. Highly sulfated HS interacts with HSV-1 glycoprotein C and glycoprotein B, and is essential for virus binding to the cells. It has also been shown that a specific 3-*O*-sulfated HS interacts with glycoprotein D which is critical for entry of the virus. Therefore HS might play various roles in mediating viral infections and the detailed mechanisms are still under extensive investigation.

Among the picornavirususes, foot and mouth disease virus type O tissue cultured variant was the first virus to be identified using HS as an alternative receptor in addition to integrin, the natural receptor for this virus (Jackson et al., 1996). However, interestingly these variants could not cause virulence in their natural host via HS and could use only integrin for productive infection. Later on, some clinical isolates of echoviruses were also shown to use HS as receptor (Goodfellow et al., 2001). Attempts to adapt major group human rhinoviruses to grow in ICAM-1 negatively cells surprisingly resulted in HS-binding variants of HRV89 (Vlasak et al., 2005a) and none of them could acquire affinity for LDLR. Furthermore two other serotypes HRV54 and HRV8 were identified which showed binding to the ICAM-1 negative rhabdomyosarcoma (RD) cells. HRV54 could successfully infect these cells although with lower efficiency but HRV8 albeit binding and entering did not show prominent CPE. Only after some alternate passages between RD and HeLa cells resulted in the selection of variants (HRV8v) that could efficiently infect and replicate in RD cells. All these viruses use HS as a receptor but at the same time preserve ICAM-1 binding ability and no one could utilize LDLR, the receptors for minor group HRVs (Khan et al., 2007).

Virus	Disease	Reference
Human pathogenic viruses		
Herpes simplex virus	Perioral and genital lesions and encephalitis	(WuDunn and Spear, 1989)
Dengue virus	Dengue hemorrhagic fever/dengue shock syndrome	(Chen et al., 1997)
Human immunodeficiency virus 1	Acquired immunodeficiency syndrome	(Tyagi et al., 2001)
Epstein-Barr virus	Burkitt's lymphoma and chronic fatigue	(Ianelli et al., 1998)
Cytomegalovirus	Kaposi's sarcoma	(Compton et al., 1993)
Vaccinia virus	Smallpox	(Chung et al., 1998)
Human papillomavirus	Cervical cancer and genital warts	(Giroglou et al., 2001)
Hepatitis C virus	Liver cancer and cirrhosis	(Garson et al., 1999)
Respiratory syncytial virus	Respiratory infections in infants and young children	(Feldman et al., 2000)
Human rhinovirus type 54	Major cause of common cold	(Khan et al., 2007)
Human rhinovirus type 8	Major cause of common cold	(Unpublished data)
Human rhinovirus type 89	Major cause of common cold	(Vlasak et al., 2005a)
Echoviruses	Fabrile and aseptic meningitis in children	(Goodfellow et al., 2001)
Varicella zoster virus	Chickenpox and shingles	(Jacquet et al., 1998)
Adenovirus	Acute respiratory infections in human and used as vector for gene therapy	(Brynes and Griffin, 1998)
Adeno-associated virus type 2	Nonpathogenic virus used as vector in gene therapy	(Dechecchi et al., 2000)
Animal pathogenic viruses		
Sindbis virus	Fatal disease in neonatal and adult mice	(Summerford and Samulski, 1998)
Foot and mouth disease virus	Highly contagious disease of hoofed mammals	(Jackson et al., 1996)
Swine fever virus	Infections of pigs	(Hulst et al., 2000)
Pseudorabies virus	Pigs infection	(Trybala et al., 1998)

Table 1.3: HS-binding viruses. Viruses almost from every group or class have shown interactions with HS and can use this ubiquitous glycoprotein as receptor or co-receptor.

Heparan sulfate 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 adhesion, cell-cell adhesion, cell proliferation, motility and differentiation, blood coagulation, inflammation, tumor progression and invasion, tissue regeneration, lipoprotein metabolism and pathogenic infections (Kuberan et al., 2002). HS belongs to a unique class of macromolecules known as glycosaminoglycans which are linear polysaccharides and in

addition to HS also include chondroitin sulfate (CS), keratin sulfate and hyaluronic acid. HS is present in the form of heparan sulfate proteoglycans (HSPG) on the surface of the cells.

Proteoglycans are composed of a core protein and polysaccharide side chains. The core proteins serve as anchors for polysaccharide chains on the surface of the cells where polysaccharides interact with the protein to execute biological functions. There are two types of core proteins namely syndecans and glypicans. Notably syndecans contain both HS and CS polysaccharide side chains while glypicans carry only HS side chain (David et al., 1990). There are four isoforms of syndicans which have conserved cytoplasmic tail but differ in amino acid sequence in the extracellular domains. The syndecan core protein family exhibits a very peculiar distribution among different cells, tissues, and at various developmental stages (Kim et al., 1994a). Furthermore, the extracellular part of the core proteins possesses several protease cleavage sites and selective cleavage results in the shedding of polysaccharides from the cell surface. Therefore, this is believed that shedding controls the level of proteoglycans on the cell surface and in the matrix and thus regulates the biological functions. Although the exact mechanism is still not clear but shedding could play very important roles for the recruitment of growth factors for tissue repair and wound healing, as well in the host response to infections by bacteria and viruses. However, it still remains to be investigated whether the level of core protein or the degree of shedding affects the susceptibility of a specific tissue to viral infections. Apart from controlling biological functions via polysaccharide side chains, isoform 4 of the core protein syndecan is directly involved in cell adhesion process (Woods and Couchman, 1994). Moreover, the intracellular domain of syndecan possesses tyrosine and serine phosphorylation sites indicating that the core protein contributes to cellular signal transduction (Horowitz and Simons, 1998; Ott and Rapraeger, 1998; Volk et al., 1999).

Glypican is another core protein, member of the HSPG family with at least 6 isoforms. In contrast to syndecan, glypican is linked to the plasma membrane via glycosylphosphatidylinositol anchors and its extracellular domain carries 14 conserved cysteine residues suggesting a compact and globular conformation due to disulfide-bridges. Glypican is highly expressed in the central nervous system, picturing its importance in neurological functions (Bandtlow and Zimmermann, 2000).
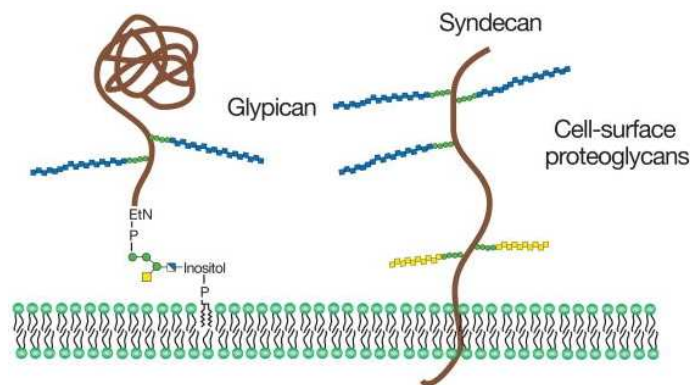
Figure 1.8: Cell surface proteoglcans. Types of proteoglycans depend on mainly core protein and side chains. Mostly they are attached to the membrane via transmembrane protein but some are conneted via GPI anchored proteins.

Polysaccharide side chains are covalently linked to the core proteins and exhibit considerable variety depending on the sequence and type of the disaccharide repeating units. HS is a copolymer of glucuronic or iduronic acid and glucosamine with various degrees of sulfation. Together with heparin, HS is marked by a linear chain of 50-200 disaccharide units of β-D-glucosamine (GlcN) linked (1→4) to β-D-glucuronic acid (monosaccharide building blocks are depicted in figure 1.9). The β-D- glucosamine can be either N-sulfated (GlcNS) or N-acetylated (GlcNAc), subsequently both may be 6-*O*-sulfated (GlcNS(6S) or acetylated (GlcNAc(6S)), or the GlcNS and GlcNS(6S) can also be 3-*O*-sulfated (GlcNS(3S) and (GlcNS(3,6S)). Furthermore, the β-D-glucuronic acid (GlcA) can be epimerized to α-L-iduronic acid (IdoA) (Rabenstein, 2002). Thus, the complexity of HSPGs is even greater than that of proteins and is thereby not only the most acidic but also the most information-dense biopolymer found in the nature (Nugent, 2000).
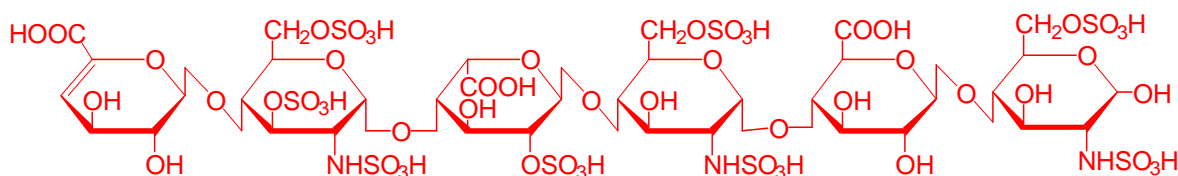
Figure 1.9: The structure of polysaccharide chain of HS.

Chondroitin sulfates are copolymers of glucuronic or iduronic acid and *N*-acetylated galactosamine with variable sulfations. For example CS A contains galactosamine 4-*O*-sulfate while CS C has 6-*O*-sulfate. Chondroitin sulfate B (also called dermatan sulfate) possesses a higher content of iduronic acid moieties. Keratin sulfate is composed of N-acetylated glucosamine and galactose whereas Hyaluronic acid is a copolymer of glucuronic acid and glucosamine. Linkage types ($\alpha(1\rightarrow4)$) versus ($\beta(1\rightarrow3)$) and the composition of the monosaccharide (glucosamine vs galactosamine) both result in remarkable variability in biological functions among HS, CS and HA.

The biosynthesis of proteoglycans is genetically regulated and unlike other proteins, their genetic information is encoded in multiple genes because they are hybrid macromolecules comprised of proteins and polysaccharides. Thus one group of genes codes for core proteins and another group for the enzymes involved in HS biosynthesis. It is obvious that HSPG biosynthesis is regulated by the availability of both core proteins and HS biosynthetic enzymes. There are multiple isoforms of sulfotransferases with distinct tissue distribution.

Interaction of the HSPGs with viruses

Approximately more than 100 proteins have been shown to interact with HS. It is obvious that the highly negatively charged HS binds to a cluster of positively charged residues on the proteins as sulfate groups are negatively charged under physiological pH. Thus, the nature of interaction mainly includes ionic, but hydrogen bonding and hydrophobic interactions also play an important role. However, the exact mechanism is still under intensive investigation and the nature of the interaction between proteoglycans and proteins might be far more complex than their structure. Two consensus sequences XBBXBX and XBBBXXBX (where B signifies residues like lysine, arginine and histidine; X represents a hydropathic residue) were proposed as heparin/HS binding motifs by Cardin and Weihtraub (Cardin and Weintraub, 1989). These motifs were then found in many other heparin-binding proteins and removing or replacing these consensus sequences could eliminate their interaction with HS (Hileman et al., 1998). However, it should be noted that there are many proteins including HS sulfotransferases, which do not have these motifs, indicating the complexity of the molecular mechanism of HS-protein interactions. It is speculated that HS interacts with proteins also

through hydrophobic and hydrogen bonds to achieve its binding specificity. HS indeed interacts through hydrogen bonding or hydrophobic interaction with fibroblast growth factor and a very similar observation was made for antithrombin with heparin pentasaccharide.

The Interaction between virus and HS has been extensively studied for herpes simplex virus type 1 (HSV-1). It was demonstrated that the virus envelope glycolprotein C and in some cases glycoprotein B are responsible for binding to HS. It is interesting that in L-cells, mutant in HS biosynthesis, chondroitin sulfate assists HSV-1 infection. CHO wild type and swine testis cells are resistant to HSV-1 infection albeit the virus shows a normal binding to these cells. It was suggested that the virus needs some entry receptor in addition to binding to the HS. Indeed, a specific 3-*O*-sulfated HS was identified that serves as entry receptor and interacts with virus glycoprotein D (Shukla et al., 1999). CHO cells do not express 3-*O*-sulfated HS and thus are resistant to HSV-1 infection. However, it was convincingly demonstrated that glycoprotein D recognizes a specific disaccharide sequence. The exact disaccharide sequence that acts as an entry receptor is still unknown. Recent data revealed that HS also plays various roles in assisting HIV infection. It has been shown that HS interacts with HIV glycoprotein (gP) 120 where, a potential heparin-binding domain with basic residues was found (Roderiquez et al., 1995). It is worth noticing that HS has different roles in mediating HIV infection depending on the type of co-receptors used by the virus. HS also facilitates the entry of HIV transactivator protein Tat.

When considering picornaviruses, a crystal structure of FMDV complexed with heparin is available. It revealed that the heparin-binding site was within a shallow depression at the junction of the three major capsid proteins and that it was distant from the integrin-binding region (Fry et al., 2005). The contact residues are the basic and polar like Arg and Asn of VP3, Thr and Arg of VP2 and Lys of VP1, together with bridging water molecules. Again, albeit there are no typical HS-binding motifs on the surface of the virus, the overall negative and polar charged patches result in the specific interaction with heparin. Human rhinovirus type 89 variants which were adapted to grow in HEp2 cells had mutations at their surfaces that rendered them more basic. However, similarly to heparin-binding FMDV, HRV89 viral isolates binding to HS lack a particular heparin recognition sequence (Vlasak et al., 2005a). Scanning of VP1 of HRV54, another rhinovirus type that uses HS for infection, revealed the

presence of a pattern of residues HHFK at the BC loop. Interestingly, a similar pattern was also found in HRV62, -65, -83, and -98 at a comparable position but was absent in HRV8 that can also bind to HS. Since the three-dimensional structure of these types is not available, it is not known whether this motif in HRV54 is sufficiently accessible (Khan et al., 2007). All these findings suggest that the presence of specific HS-binding motifs or just charge complementarity plays a major role in interaction of HS with their ligands.

Entry mechanisms

The plasma membrane serves as a barrier and separates the extracellular milieu from the interior of the cell. Where small neutral molecules can diffuse across the membrane, large molecules and pathogens require special mechanisms to cross this barrier. Viruses being obligate cellular parasites must get access to the interior of the cells to carry out their life cycle. Perhaps, the simplest way of entry is via direct fusion with the plasma membrane. Indeed, this route of entry is used by different viruses and bacterial toxins. However, majority of viruses bind to specific receptors on the surface of the cell which lead to entry and intracellular trafficking of the virus. These viruses exploit and hijack natural entry portals used by cargos under physiological conditions. Generally, virus entry after binding to a particular receptor is most often termed as "receptor mediated endocytosis." However, recently Gary J. Doherty and Harvey T. McMahon have urged to abandon this term because methods are readily available to dissect the type(s) of endocytosis used by the virus of interest (Doherty and McMahon, 2009). There seems to be at least eight different pathways by which viruses can be internalized, but many of them may be manifestations of the same pathway, either being modified by the viruses themselves or dependent upon the specific cell type (Marsh and Helenius, 2006).

Nevertheless, virus entry can be dissected into four distinct endocytic pathways which are comparatively well understood: (1) clathrin-mediated endocytosis (CME), (2) the caveolin dependent pathway, (3) non-clathrin non-caveolae pathway and (4) macropinocytosis. Each entry pathway is characterized by the involvement of certain key components and an array of accessory proteins (figure 1.10).
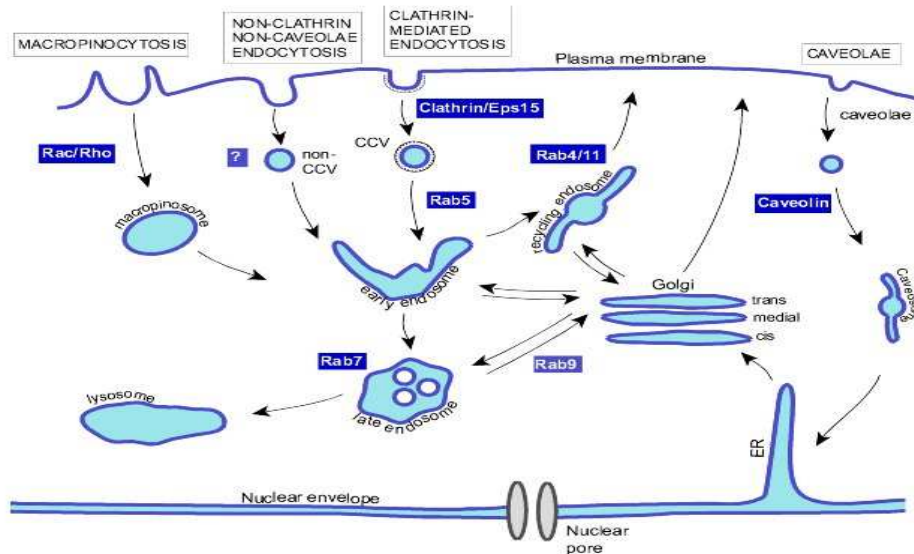
Figure 1.10: An overview of various entry pathways. Each entry pathway is characterized by the key ptotein, vesicle size and structure, intracellular trafficking and final destination of cargo taken up by a particular pathway. Different Rab proteins are involved in segregation and transport of cargo. CCV stands for clathrin carrier vesicle.

Clathrin-mediated endocytosis (CME)

Endocytosis controls the protein and lipid constituents of the plasma membrane, thereby regulating how cells interact with their environment. Endocytosis is also involved in the regulation of numerous processes, including nutrients uptake, signaling, cell adhesion and migration, synaptic transmission, receptor down regulation, pathogen entry, antigen presentation, cell polarity, mitosis, growth and differentiation, and drug delivery. Clathrin-mediated endocytosis is probably the best understood pathway hitherto albeit the exact role of various accessory proteins under different circumstances is still not clear. Proteins involved in CME recruit the cargo into developing clathrin-coated pits (CCPs), and subsequently make clathrin-coated vesicles (CCVs). Clathrin, the key protein of CME discovered 29 years ago, forms the remarkable lattices which surround CCPs and CCVs. It is a spider like molecule, with three legs radiating from a central hub (Ungewickell and Branton, 1981). Each leg contains a 190 kDa heavy chain and an associated 25 kDa light chain where antiparallel

interaction of the legs from triskeletons centered on the adjacent vertices results in assembly of a coat (Kirchhausen and Harrison, 1981). The formation of CCPs is triggered by the signal from the cytoplasmic tail of a receptor involved in the cargo. Well known clathrin clustering signal sequences are YXXφ and di-leucine. Clathrin also interacts with a number of other indispensable molecules called adaptor or accessory proteins. These include AP-2 (adaptor protein-2), Eps 15 (epsin 15), SNX9 (sorting nexin 9), actin, actin-associated proteins N-WASP, Arp2/3 ARH and Dab2, amphiphysin, AP180, dynamin and many others. AP-2, the first identified clathrin adaptor is able to link clathrin in cargo and serves as a hub for protein-protein interaction, essentially clustering numerous proteins at the site of CCVs formation (Schmid et al., 2006). AP-2 can also bind to PtdIns(4,5)P_2 which is required for the proper clathrin localization, as well as progression of endocytosis (Beck and Keen, 1991; Zoncu et al., 2007). However, there are currently a number of different perspectives on whether AP-2 has a functional role as cargo adaptor within the nascent vesicle or whether it is mainly involved in cargo sorting at the cell surface. The role of dynamin 1 and 2 has been very well established in CME (Altschuler et al., 1998). Although dynamin-1 is expressed only in cells from neuronal lineage, dynamin-2 is found ubiquitously (Cao et al., 1998). Dynamin is a large GTPase and mediates membrane scission upon GTP hydrolysis thus irreversibly pinching off the vesicles from the plasma membrane and releasing them into the interior of the cell (Praefcke and McMahon, 2004). Dynamin-1 preferentially binds to the PtdIns(4,5)P_2 which promotes dynamin oligomerization, a process regulated by other curvature-sensing proteins like the BAR domain possessing proteins SNX9 and amphiphysin1 (Ramachandran and Schmid, 2008). There are some conflicting reports about the recruitment of dynamin from the cytosol to the site of scission and its gradual internalization and rapid disappearance from the cell surface. Thus it does appear as dynamin might have a role beyond the vesicle neck. Although dynamin is not directly associated with actin, several dynamin-binding proteins can interact with actin or with proteins that regulate actin assembly, thereby coordinately regulating actin assembly and trafficking events. This might establish the connection of actin with endocytic events. Epsin is another member of a family of proteins which have shown to play a role in inducing membrane curvature and acts as an adaptor for ubiquitinated cargo (Ford et al., 2002; Hawryluk et al., 2006). Thus epsin provides an initial membrane deformation in concert with clathrin polymerization to form a clathrin bud (Hinrichsen et al.,

2006). Apart from the above accessory proteins playing an active part in CME directly or indirectly, recent findings have shown that proteins that were originally shown to be important regulators of more diverse cellular phenomena, such as apoptosis (p53), asymmetric cell division (numb), and cell polarity (par proteins) are also intimately involved in CME (Ohmori et al., 2008; Santolini et al., 2000).

Investigation of a particular entry pathway has been promoted by the use of a number of most recent and handy techniques and by exploiting specific markers. Amongst them are electron microscopy (morphology of vesicles), fluorescent microscopy (partner proteins), TIR-FM (total internal reflection fluorescent microscopy; real time behavior of tagged proteins), siRNA, DN-proteins (dominant negative proteins; key or accessory proteins) and pharmacological inhibitors to just name a few. Natural cargos taken up by CME are the transferrin receptor, receptor tyrosine kinase (RTKs), G protein-coupled receptors (GPCRs) and anthrax toxin, amongst many others. Researchers in this field have made use of these markers to understand subtle details of the mechanism. Although depletion of clathrin and AP-2 shows a profound effect on transferrin entry, a great caution should be taken to interpret the results as the behavior of the markers can vary under different circumstances. Nevertheless, overexpression of the C terminus of AP180 has been shown unequivocally to inhibit CME. Albeit their potential side effects and poor specificity, many pharmacological drugs are still extensively used to dissect various entry pathways. Chemical inhibition of clathrin-dependent internalization can be traced back in the early 1980s when monodansylcadaverine (MDC), potassium depletion, and hypertonic sucrose were introduced to block receptor-mediated endocytosis (Daukas and Zigmond, 1985; Davies et al., 1980; Larkin et al., 1983). Chlorpromazine, a cationic amphipathic drug, when used in micromolar concentrations inhibits CME of various plasma membrane proteins (Inal et al., 2005). NH_4Cl (a weak base that changes cytosolic pH) and phenylarsine oxide (PAO) are other well known CME inhibitors (Cosson et al., 1989; Gibson et al., 1989). Despite the fact that none of the above inhibitors possesses absolute selectivity, some of these chemicals and/or treatments, such as potassium depletion, chlorpromazine, and MDC, can be used for the initial discrimination between clathrin-mediated internalization and other endocytic pathways (a comprehensive article on the various drugs used for different entry pathways and their potent side-effects has been recently published by Andrei I. Ivanov, 2009).

30

Clathrin-mediated endocytosis in virus entry

Being the major endocytic entry pathway, clathrin-mediated endocytosis has been exploited by a number of viruses. However, it should be noted that viruses mainly induce their own CCVs rather than using the preexisting ones. Traditionally, based on transmission electron microscopy, many viruses like influenza virus, SFV, and VSV were identified in clathrin coated vesicles at an early time of internalization (e.g., 5 min) (Marsh and Helenius, 1980; Matlin et al., 1981, 1982). However, noncoated vesicles were also found in these experiments either due to already release of clathrin or the presence of clathrin independent pathways. Although a role of CME in VSV and SFV entry has more recently been confirmed by the expression of DN-Eps15 (that arrests CCPs assembly at the cell surface), the mechanism of influenza virus entry is still far less clear (Sieczkarski and Whittaker, 2002b). For example 60% of influenza viruses do enter through CME, the remainder enter via a clathrin- and caveolin-independent route (Rust et al., 2004). Thus, influenza virus may have the ability to exploit clathrin-dependent and –independent entry pathways and there might be a compensation for the loss of a particular uptake mechanism by another (Damke et al., 1995). Adenovirus, sindbis virus, parvovirus, and Hantaan virus are among the other viruses whose entry requires functional clathrin (Carbone et al., 1997; Jin et al., 2002; Parker and Parrish, 2000).

Because of much conflicting data, picornaviruses are among many other virus families where the role of clathrin is not well established. Poliovirus has been proposed to enter independently of clathrin, caveolin, flotillin and macropinocytosis, but entry is tyrosine kinase and actin dependent and release of genome occurs close to the plasma membrane (Brandenburg et al., 2007; DeTulleo and Kirchhausen, 1998). FMDV enters through clathrin-dependent pathway when it binds to the integrin family of receptors (O'Donnell et al., 2005). On the other hand, recently it has been shown that entry via heparan sulfate is clathrin-independent and is strongly affected in the cells where caveolin functions are impaired (O'Donnell et al., 2008). These results indicate that the entry of a virus could be linked to a specific receptor which has inherent information for particular endocytic traffic. One such example is observed in enteroviruses where entry is independent of clathrin but dependent on lipid rafts of the cell (Stuart et al., 2002) and use of novel pathway is directly related to the type of the receptor used (in this case DAF). A role of clathrin in echovirus-2 entry was

shown but the virus was trafficked to the ER instead of traditional route of CME to endosomes (Joki-Korpela et al., 2001). On the other hand, echovirus-1 shows no colocalization with clathrin markers and appears to use a caveolin dependent pathway (Marjomaki et al., 2002). However, it has recently been shown that the majority of early virus uptake does not occur through caveolae and that $\alpha_2\beta_1$ integrin clustering defines its own entry pathway which is Pak1 dependent but clathrin and caveolin independent and can sort cargo to caveosomes (Karjalainen et al., 2008).

Human rhinoviruses use two different receptors and hence could use more than one entry pathways. Minor group HRVs as exemplified by HRV2 bind to LDLR which has clathrin clustering signal. Potassium depletion has shown a role for clathrin dependent entry for HRV2 albeit with pleiotropic effects observed in these experiments (Bayer et al., 2001; Madshus et al., 1987). However, later on using DN-protein inhibitors and dynamin K44A expression, it was convincingly shown that HRV2 enters HeLa cells via a cholesterol- and clathrin-dependent manner (Snyers et al., 2003). On the other hand, majority of rhinoviruses bind to ICAM-1 and entry pathway has not been revealed in detail. Based on inhibition of infection by the dominant negative dynamin-2 mutant K44A, it was proposed that HRV14 also follows a clathrin-dependent pathway (DeTulleo and Kirchhausen, 1998). However, ICAM-1 lacks a typical clathrin localization signal and even functions as viral receptor when its cytoplasmic tail is replaced with a GPI-anchor (Staunton et al., 1992). Therefore, involvement of clathrin in HRV14 uptake appears unlikely as dynamin-2 has been also shown to be important for other entry pathways. Furthermore, a specific entry pathway for ICAM-1 ligands has been identified where uptake was found to be triggered on binding of multivalent ligands, such as immunobeads, and occurred independently from clathrin and caveolin. Inhibition by amiloride, actin-depolymerization, and protein kinase-C inhibitors pointed to macropinocytosis (Muro et al., 2003). However, recently Lau C et al has shown recruitment of the normally cytosolic Syk to the plasma membrane upon HRV16-ICAM-1 binding, along with Syk-clathrin coassociation. Upon internalization Syk localized to punctate structures resembling endosomes and colocalized with HRV16 (Lau et al., 2008). Thus in case of rhinoviruses, it may be that different serotypes use different pathways (Sieczkarski and Whittaker, 2002a). It is of note that there are certain HRV types that exhibit dual receptor specificity and can utilize HS in addition to ICAM-1 (Khan et al., 2007; Vlasak et al., 2005a).

We have shown that HRVs entry via ICAM-1 and HS is clathrin, caveolin and flotillin independent and that it most probably follows dynamin-independent in the former and dynamin-dependent macropinocytosis in the latter case (Khan et al., 2009; submitted).

Entry via Caveolae

In contrast to clathrin-mediated endocytosis where the understanding of the regulatory machinery and structure of individual CCPs has dramatically progressed, the role of caveolae as an alternative endocytic pathway still remains elusive. Caveolae were first reported as smooth-surfaced flask shaped pits ranging typically between 50 and 65 nm in diameter (Yamada, 1955). Identification of the major constituent protein of caveolae, the caveolin1, yielded the first molecular marker of caveolae and boosted the progress of elucidation of their formation and function (Rothberg et al., 1992). The confirmation of the involvement of caveolin1 in caveolae formation came from the discovery that lymphocytes lacking caveolin1 expression did not show morphological caveolae, and transient expression of this protein in these cells was sufficient to give rise to the characteristic structures identifiable by EM (Fra et al., 1995). There are three mammalian caveolin proteins. Whereas caveolin3 is muscle specific, caveolin1 and 2 are expressed widely in non-muscle cells with exception of neurons and leukocytes which apparently lack caveolae. The caveolins belong to the family of integral membrane proteins consisting of a putative 33 amino acid central hydrophobic domain flanked by cytoplasmically exposed C- and N-terminal domains (Dietzen et al., 1995; Monier et al., 1995). Caveolin1 possesses palmitoylated C-terminal domain which interacts directly with cholesterol and fatty acids forming high molecular weight oligomers (Monier et al., 1995). This property is believed to be important for caveolae formation although the exact mechanism involved is still not clear. This shows the involvement of lipid rafts of the membrane in the caveolae pathway although rafts have been demonstrated to play a role in other clathrin-independent pathways as well (Kirkham and Parton, 2005). Lipid rafts are conceived as islands of highly ordered saturated lipids and cholesterol more tightly packed than the surrounding bilayer, that float freely in the membrane (Simons and Ehehalt, 2002). Thus, they are relatively resistant to non-ionic detergents and this property can be used as a marker for lipid rafts association. As cholesterol is the key partner of the lipid rafts and their properties virtually depend on the concentration of cholesterol in the rafts, drugs that

sequester or remove cholesterol have been used to understand the role of rafts and their bona fide proteins (Foster et al., 2003). Cholesterol perturbation flattens the caveolae which increases the caveolin1 mobility in the plasma membrane (Rothberg et al., 1992). Proteins found within lipid rafts in addition to caveolin1 are glycosylphosphatidylinositol (GPI)-anchored, dually acylated proteins (e.g. the Src family tyrosine kinases and Gα subunits of the heterotrimeric G proteins) and cholesterol associated Sonic hedgehog etc (Simons and Toomre, 2000). Recently a total of 80 different kinases and various signaling molecules have been identified that are somehow associated with membrane microdomains and are involved in caveolar cargo transport as exemplified by simian virus-40 entry (Pelkmans and Zerial, 2005).

Caveolae as a constitutive endocytic pathway is still controversial mainly due to a normal endocytic process in caveolin1 lacking cell types. Furthermore, a non-caveolar pool of the putative caveolar markers could be internalized rapidly via clathrin-mediated endocytosis. This makes remarkably difficult to study the caveolae pathway. A recent study has revealed that caveolin1 shows homology to GDI sequence and binds to GDP-bound cdc42 which is strongly implicated in GLIC/GEEC endocytosis. Thus caveolin1 manipulations may also modulate other pathways. It is also noticeable that the caveolar pathway is inducible and can be stimulated in the presence of cargo (Tagawa et al., 2005). Indeed, addition of GSL (glycosphingolipid) and elevated levels of cholesterol in human skin fibroblasts resulted in the stimulation of endocytosis of caveolar markers without affecting other clathrin-dependent and CI pathways (Sharma et al., 2005). Nevertheless, endocytosis via caveolae in the majority of the cells shows characteristics which are very different from that mediated by CCPs.

Very few markers have been shown to be internalized through the caveolin1-dependent pathway and perhaps transcytosis across the endothelial barrier is currently the most convincing trafficking process associated with caveolae (Heltianu et al., 1989). Some of the other cargos are cholera toxin subunit B (CTxB), SV40 and GI anchored proteins, although CTxB and SV40 can also use other pathways. CTxB belongs to a family of bacterial toxins and its B subunit binds to a glycolipid receptor, the GMI ganglioside (Fishman and Orlandi, 2003). Early studies revealing labeling of caveolae with CTxB-gold made this toxin favorite marker for the caveolae (Montesano et al., 1982). However, cells lacking the expression of caveolin1 are also sensitive to this toxin (Orlandi and Fishman, 1998). Thus, caveolae are

clearly not essential for the CTxB internalization. Rather, a lipid raft dependent entry might lead to toxicity by this protein (Wolf et al., 1998).

A variety of pharmacological inhibitors are known to interfere with the raft-dependent caveolae endocytic process by targeting cholesterol (Parton and Richards, 2003; Smart and Anderson, 2002). However, cholesterol targeting can significantly alter pathways other than caveolae although these effects are mostly cell type specific. Statins are a family of drugs which inhibit cholesterol production by irreversibly binding to a rate limiting co-enzyme involved in cholesterol biosynthesis (Liao and Laufs, 2005). Methyl-β-cyclodextran (MβCD), one of the widely used inhibitors, is a heptasaccharide possessing hydrophobic core which interacts with cholesterol and hence easily extracts the cholesterol from the membrane (Irie et al., 1992). Polyene antibiotics such as filipin and nystatin readily sequester the cholesterol and create large aggregates at the membrane (Kitajima et al., 1976). While, MβCD inhibits CME and raft-dependent macropinocytosis, the effects of the latter drugs especially that of filipin are much specific on caveolar endocytosis (Monis et al., 2006; Smart and Anderson, 2002).

The caveolar pathway in virus entry

Simian virus-40 (SV40) is probably the best known marker of the caveolar pathway. However, subsequent trafficking of SV40 is somehow unusual as the incoming virions accumulate in the smooth ER (Kartenbeck et al., 1989). SV40 uses the major histocompatibility complex (MHC) class 1 molecules as primary receptor as demonstrated by inhibition of virus adsorption and subsequent internalization by specific antibodies to this protein (Stang et al., 1997). Entry via caveolae was shown by virus colocalization with caveolin and more recently with GFP-tagged caveolin1 combined with video microscopy. It demonstrated the delivery of SV40 from caveolae to the ER, with the involvement of dynamin and actin, but by passing the traditional endosomal/lysosomal route (Norkin, 1999; Pelkmans et al., 2001; Pelkmans et al., 2002). Other notable peculiarities of the SV40 entry through caveolae are the slow kinetics as compared to CME, the vesicles do not become acidified, and internalization is an active process induced by virus binding. However it should be noted that SV40 entry went on unaffected in caveolin1-null cells and the virus uptake was faster when compared to caveolin1 positive cells and was not altered by the overexpression of dynamin2 K44A or DN-arf6 (Damm et al., 2005). Consequently, the pathway was proposed to be

clathrin and caveolin independent but cholesterol sensitive. Entry via caveolae has also been suggested for mouse polyoma virus, however, it was found that incoming virus did not colocalize with caveolin (Gilbert and Benjamin, 2000).

Among the picornaviruses, EV1 was reported to exploit this route but recently it has been shown that the receptor used by this virus defines its own entry pathway. At least in the cells used in this investigation, entry was clathrin and caveolin independent (Karjalainen et al., 2008; Marjomaki et al., 2002). A caveolar pathway has also been implicated in heparan sulfate binding FMDV but how this virus would release its genome in the absence of a low pH trigger is not clear. Furthermore we have most recently shown that a rhinovirus that uses HS for binding enters via macropinocytosis (manuscript submitted).

Macropinocytosis

Macropinocytosis is defined as a series of events accompanied by the extensive reorganization or ruffle of the plasma membrane to shape external macropinocytic structures which are then enclosed and internalized into the cell. It is a general feature of macropinocytosis that the active portion of the plasma membrane is initially not involved in invagination but rather an actin-dependent protrusion of the membrane to the external milieu. Furthermore, vesicles derived from this process (macropinosome) are of variable size and may be several micrometers in diameter, significantly larger than CCVs or caveosomes. Macropinocytosis shares many features with phagocytosis and both processes are much confined to specific cell types such as macrophages and dendritic cells. However, macropinocytosis has also been demonstrated to be prominent in cell types that do not phagocytose at all. It occurs mainly upon stimulation by growth factors rather than being a constitutive event (Hewlett et al., 1994). Constitutive macropinocytosis has been observed in NIH3T3 fibroblasts expressing a ruffling kinase in addition to dendritic cells but whether it is an essential cellular activity in non-phagocytic cells has to be established. Another intriguing feature of macropinocytosis is that clathrin-independent endocytic processes which are constitutive under cell rest conditions can divert to this process upon stimulation. Addition of growth factors initiates membrane ruffling, resulting in the rapid increase in uptake of fluid phase markers (Chinkers et al., 1981; Haigler et al., 1979). However, it is interesting to note that treatments which inhibit membrane ruffling have no effect on growth factor stimulated

macropinocytosis suggesting that ruffles themselves are not essential for this process (Li et al., 1997). Two markers, fluorescent-dextran and horseradish peroxidase, have been extensively used in fluorescent and electron microscopy to investigate the process of macropinocytosis.

Albeit macropinocytosis is a comparatively well established process, its regulation and the proteins involved are still largely elusive. Macropinocytosis has been heavily implicated with the use of small GTPases, such as the Rac/Rho family and Arf family members (Arf6) instead of the vesicles scission protein dynamin. Many other signaling molecules such as Ras, Src, PAK1, and phosphatidyinositol kinases (PIK) play a major role in stimulation and regulation of this process. The kinase PAK1 which binds to rac1 has been exclusively associated with macropinocytosis and is indispensable for, and sufficient to induce, this process (Dharmawardhane et al., 2000; Knaus et al., 1998). Furthermore, activation of PI3K, rac, and src stimulate this process (Amyere et al., 2000; Gao et al., 2007; Veithen et al., 1996). Macropinocytosis is cholesterol sensitive because this lipid is required for the recruitment of activated rac1 to the ruffling sites. Rufflings are also enriched with membrane microdomain markers suggesting the dependence of this process on the lipid rafts (Grimmer et al., 2002; Manes et al., 2003). Apart from the above mentioned small GTPases, Rab proteins have been shown to play a major role in macropinocytosis. Rab5 which is also implicated in other endocytic pathways, when expressed along with Ras promotes the formation of circular ruffles and its DN-mutant (S34N) inhibits circular but not cell edge rufflings (Lanzetti et al., 2004). The role of Rab5 was further confirmed by partial silencing of one of its effectors, Rabankyrin-5, which resulted in the diminishing of EGF-stimulated fluid phase uptake (Schnatwinkel et al., 2004). Other Rab proteins, which have been implicated in this process are Rab 21, Rab22a, and Rab34 (Sun et al., 2003).

Despite the fact that macropinocytosis and phagocytosis are the best known types of endocytosis, the number of pharmacological inhibitors of these processes are very few. In fact, there are three major types of drugs which have been used to block macropinocytosis and phagocytosis. They include sodium–proton exchange inhibitors, F-actin-depolymerizing agents, and drugs that target the phosphoinositide metabolism. Amiloride (an Na/K ion-exchanger) and its derivatives 5-(N-ethyl-N-isopropyl) amiloride (EIPA) and dimethyl amiloride (DMA) have been shown to inhibit constitutive and stimulated macropinocytosis in

a variety of mammalian cells (Nakase et al., 2004; von Delwig et al., 2002). Since macropinosomes are characterized as large F-actin-coated vesicles, drugs that depolymerize the F-actin cytoskeleton significantly impair vesicle formation and subsequently macropinocytic endocytosis. Cytochalasin D and Latrunculins have been shown to inhibit macropinocytosis by disrupting actin assembly. Cytochalasin D inhibits actin polymerization by blocking the faster-growing "barbed" end of actin filaments, whereas latrunculins bind to monomeric actin, thus preventing its incorporation into filaments (Peterson and Mitchison, 2002). Two drugs, wortmannin and LY290042, are known as PI3K inhibitors and have been reported to interfere with constitutive and stimulated macropinocytosis in various cell types (Araki et al., 1996; Dharmawardhane et al., 2000).

Macropinocytosis in virus entry

Macropinocytosis is generally conceived as a non-specific entry process because of its involvement with the ligands which are not associated with specific receptors. Instead, endocytic vesicle formation is triggered by stimulation of the cell, particularly at the site of ruffling. The finding that macropinosomes have the ability to become acidified and can intersect endocytic vesicles made them a plausible route of entry for a variety of virus types. Macropinocytosis has been implicated in the entry of HIV-1 in macrophages, although the majority of the virus internalized by this way was non-infectious (Marechal et al., 2001). A role of macropinocytosis in virus induced endocytosis has been proposed for adenovirus type 2 despite the fact that the virions themselves are internalized in CCVs (Meier et al., 2002). Among picornaviruses, we have recently shown that HRVs entry via ICAM-1 and HS depends on macropinocytosis in rhabdomyosarcoma cells. Interestingly, in the former case no dynamin functions were required whereas in the latter case entry was sensitive to dynasore, a dynamin inhibitor (manuscript submitted). Apart from virus entry, an appreciable set of data suggests that macropinocytosis has a major role in the entry of various membrane penetrating peptides. One of the best known peptide is the TAT from HIV, which binds to cell surface heparan sulfate and is being internalized through macropinocytosis. However, for many other HS-binding peptides, the entry mechanism is much more complicated and might depend on the concentration of the peptide and type of the cell (Duchardt et al., 2007). However recently

it was shown that the eosinophil cationic protein enters via HS and uses raft dependent macropinocytosis (Fan et al., 2007).

Viral uncoating

After binding to surface receptors and subsequent internalization, viruses release their genome at or close to the site suitable for replication/translation. Uncoating is associated with the structural changes in the capsid of the virion either caused by low pH milieu or receptor binding which results in disruption of the capsid or opening of a pore through which genomic material can be released. Human rhinovirus infectious virions (sedimenting at 150S) uncoat through noninfective intermediate particles (135S), which are characterized by the loss of VP4 and the externalization of the hydrophobic N-termini of VP1, making the capsid hydrophobic. These intermediate particles further evolve into empty capsids (80S) by the release of the RNA (Price et al., 1996). ICAM-1, the receptor of major group HRVs is able to catalyze this conversion process. A truncated ICAM-1 containing all 5 Ig domains but lacking the transmembrane region (sICAM-1) has been shown to possess antirhinoviral activity by blocking the binding site for the cellular receptor and producing noninfectious empty particles *in vitro* (Arruda et al., 1992; Casasnovas and Springer, 1995; Greve et al., 1991). From *in vitro* experiments it was shown that uncoating is initiated from the floor of canyon and addition of WIN compounds in fact stabilize this area. In the presence of these drugs neither externalization of VP4 nor of the N-terminus of VP1 occurs (Lewis et al., 1998). Although ICAM-1 alone is necessary for, and sufficient to induce uncoating in major group HRVs, certain more stable serotypes also require low pH for efficient uncoating. For example HRV14 can be uncoated independently from low pH and infects even in the presence of bafilomycine A, whereas HRV16 requires a low pH in addition to ICAM-1 (Nurani et al., 2003). Thus ICAM-1-induced uncoating is accelerated by low pH and more stable viruses most probably can only be uncoated when both conditions are met. On the other hand, the poliovirus receptor (PV) mediates virus uncoating without the aid of low pH despite the fact that the virus is far more stable than rhinoviruses.

A two step mechanism of uncoating triggered by ICAM-1 has been proposed for major group HRVs. In a first step, the receptor binds within the canyon which results in the formation of irreversible virus-receptor complex. In a second step, a global expansion of approximately 4% in the virion occurs, which results in the expulsion of the pocket factor displacing the receptor towards the north rim of canyon. This expulsion creates a firm and tight interaction of the receptor with the virus which results in the shifting away of VP1 from the fivefold axis and destabilization of the capsid. Such drastic movements could open a 10 Å channel at the fivefold vertex through which externalization of VP4 and the N-terminus of VP1 happen, eventually RNA would be released as shown in the following schematic diagram (Hewat and Blaas, 2004).
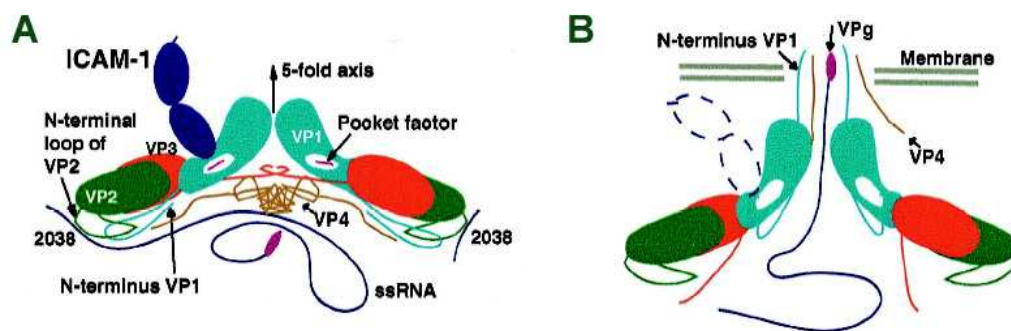
Figure 1.11: Schematic mechanism of RNA release and reorganization of the HRV14 capsid. In the native capsid (A), the RNA is bound to Trp 2038 of VP2 and the pocket is occupied with a pocket factor. ICAM-1 binds in the canyon in a two-step process which expels the pocket factor and induces a hinge-type movement of VP1 (B). Each VP1 protein is cantilevered up and away from the fivefold axis while it swivels around to open a 10 Å diameter channel to allow exit of the VP4 molecules, the N termini of VP1, and the RNA (Hewat and Blaas, 2004).

In contrast to ICAM-1, the receptors of minor group HRVs cannot trigger the uncoating process and act only a vehicle to transfer the virus to the endosomal system where the low pH alone mediates virus uncoating (Prchla et al., 1994). Thus, minor group HRVs as exemplified by HRV2, strictly depend on the low endosomal pH and uncoating occurs as soon as virus reaches the late endosomes (Brabec et al., 2006). Bafilomycin A completely prevents virus uncoating and infection can be induced from the plasma membrane provided that the

conditions prevailing in endosomes are met outside the cell (Brabec et al., 2003). As LDLR does not bind within the canyon, the receptor has not to compete with the pocket factor. Similarly to the situation with major group HRVs, at low pH (≤5.6) the pocket factor is expelled which results in the formation of a channel through which RNA can come out. These events are accompanied by the exit of N-terminus of VP1 at the junction of VP1, VP2 and VP3 while VP4 is lost through the fivefold axis. The N-terminus of VP2 bends inwards and might help to detach the RNA from its interaction with conserved tryptophans (Try 2038) (Fig. 1.12). However, no such movements were seen in HRV14 questioning whether this is a general mechanism or just a coincidence.
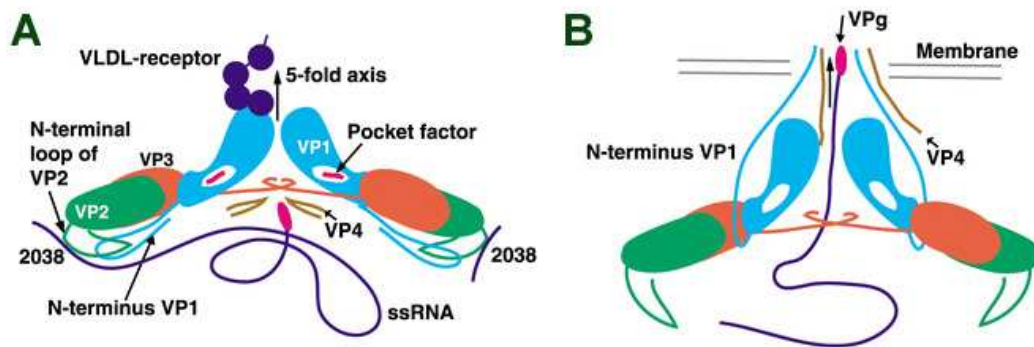
Figure 1.12: Schematic representation of the reorganization of the HRV2 capsid during uncoating. In native capsid (A), the RNA is linked to Trp2038 of VP2, and the pocket is filled with a pocket factor. The pocket factor is expelled probably in the low endosomal pH, thus providing VP1 greater flexibility (B). Each VP1 protein is cantilevered up and away from the 5-fold axis while it swivels around to open a 10 Å diameter channel allowing exit of the RNA (Hewat et al., 2002).

Certain major group HRVs, either wild type or variants use HS as an alternate receptor. These viruses have been reportedly shown to depend on the endosomal low pH for uncoating in the absence of ICAM-1 (Khan et al., 2007; Vlasak et al., 2005a). With respect to uncoating, these viruses behave essentially similar to the minor group HRVs when they bind to HS. Another major difference between minor and major group HRVs is the way, how they translocate their RNA genome across the endosomal membranes. Using 10 and 70 kD fluorescent-dextran, it has been proposed that major group HRVs tend to disrupt the endosomal membrane for

genome release and the viral proteins enter the cytosol while minor group HRVs make a pore from which only RNA can pass and thus maintain the integrity of endosomes (Brabec et al., 2005). However, the exact mechanism of RNA release and the behavior of viruses towards endosomal integrity still remain elusive.

Viral replication, assembly and release

Being positive sense RNA, the rhinovirus genome is directly translated after its release from the capsid. The small VPg protein is cleaved off by a cellular esterase and the highly structured 5'UTR recruits the small ribosomal subunits to initiate polyprotein synthesis. This 5' UTR region comprises the internal ribosomal entry site (IRES) which is peculiar to the picornaviruses but also present in HCV, HIV, RNA retroviruses and some capped mRNAs which are expressed during stress conditions (Schneider and Mohr, 2003). The initial translation of the incoming RNA results in the production of a polyprotein which is autcatalytically cleaved to produce structural and non-structural proteins which are essential for viral progeny production. The proteolysis is initiated by the $2A^{pro}$ which, through self cleavage splits off the P1 region from the P2 and P3 part. $2A^{pro}$ also targets host cellular factors such as eukaryotic initiation factor 4GI (eIF4G) and its homologue eIF4GII to shut off the capped mRNA translation. In this way, the virus can utilize all the cellular resources for its efficient production. The subsequent cleavage of each part of the polyprotein into corresponding proteins is brought about by the activity of $3C^{pro}$ (a chymotrypsin like cysteine protease as $2A^{pro}$).

RNA replication proceeds at highly ordered, rosette-like replication complexes, which include replicating viral RNA, viral and cellular proteins, and turbinated vesicles. These virus-induced vesicles play an important role in HRV RNA replication as nearly all RNA species produced during genome replication are found associated with vesicular membranes (Egger et al., 2002). The origin of these vesicular membranes is still not clear. In poliovirus-infected cells the vesicles breed either from coat protein complex II coated vesicles, which transport proteins from the ER to the Golgi (Rust et al., 2001) or from autophagosomes (Suhy et al., 2000). The very first step in the replication process is the production of negative strand RNA

which acts as a template for further virus genome multiplication. An internal RNA hairpin loop is responsible for the recognition of RNA by protein complex to initiate the minus-strand RNA synthesis. This internal cis-acting replication element (cre) is located within the VP1 region of HRV14 and within $2A^{pro}$ of HRV2 (McKnight and Lemon, 1998). VPg-pUpU binds at the poly-A end and serves as a primer for viral encoded RNA-dependent RNA polymerase ($3D^{pol}$) which is involved in the synthesis of both minus- and plus-stranded RNA. Thus a short lived intermediate complex is produced which is composed of newly synthesized minus-stranded RNA and plus-stranded input RNA. Two adenines, at the 3´ end of the nascent transcribed RNA, function as VPg-pU-pU-OH primer binding site and replication of the RNA genome can proceed via $3D^{pol}$ mediated elongation (Pathak et al., 2002). The plus strand RNA is either used as a template for translation of the polyprotein or is packaged into newly assembled provirions.

With an increasing concentration of proteins, protomers comprising one copy of each VP0, VP3 and VP1 are formed. The myristic acid linked to the N-terminus of VP0 therein plays a vital role and brings about the assembly of protomers into pentamers (Ansardi et al., 1992). These pentamers then package the VPg-RNA to produce non-infectious provirions. However, whether the packaging occurs via nucleation of the RNA or its threading into the already empty capsid is still elusive. Nevertheless, it has been reported that only actively replicating genomes can be packaged suggesting a role of replicative proteins in RNA encapsidation (Nugent et al., 1999). The non-infective provirions become infectious particles only after the maturation of the capsid which is ensued upon cleavage of VP0 into its precursors VP2 and VP4. The mature infectious virions are then released from the cells by lysis, which is observed as cytopathic effect (CPE). However, in contrast to tissue culture cells, rhinovirus infections do not cause appreciable cell damage to the nasal epithelium.

Pathogenicity of Rhinovirus Infections

Human rhinovirus infections are often considered as innocuous and that is why even at the begining of 21th century, many aspects of HRV epidemiology, immunology, clinical impact and strain characterization remained poorly addressed (Couch, 1984; Monto et al., 1987). Nevertheless, it is very clear that HRVs are a common reason for antibiotics prescription to treat respiratory illnesses and even so more than bacterial infections (Rotbart and Hayden,

2000). Furthermore, HRVs are often associated with expiratory wheezing exacerbations and thus collectively impose a huge burden on health resources at various levels (Jacques et al., 2006; Monto, 2002; Nyquist et al., 1998). HRVs were named because of their primary target, the human nose and were found to be predominantly involved in the infections of upper respiratory tract (URT). However, they occasionally also infect the lower respiratory tract (LRT)(Taylor-Robinson, 1963; Tyrrell et al., 1960). There is no identifiable pattern of HRV strains circulation (Phillips et al., 1968). However, it has been proposed that HRV-A group strains are over-represented among symptomatic respiratory infections (SRIs) than HRV-B but this could also be due to their greater number (Andries et al., 1990). Nonetheless, a more practical finding demonstrated that less HRV39 (HRV-A) was required to produce cold symptoms than HRV14 (HRV-B) in antibody-free adult volunteers (Hendley et al., 1972).

HRVs infections occur throughout the year but usually their peak is in spring and autumn (Fox et al., 1975; Miller et al., 2007; Winther et al., 2006). Clinical systems appear within 16 hours of inoculation and attain peak between 24-48 hours. Shedding of the virus is usually limited to 10-14 days in immunocompetent subjects although in some cases illness or shedding may not be detectable even after experimental inoculation (Douglas et al., 1966; Yamaya et al., 2007). Children and adults usually appear asymptomatic after the 5th day of post infection (Cate et al., 1964). Chronic infections in children commonly do not occur but picornavirus RNA has reportedly been detected via RT-PCR even at the 2nd and 3rd week after disease onset, by a time the child has been defined as asymptomatic for a week or more (Winther et al., 2006). However, truly chronic shedding (>8 months) of same HRV strain is only associated in individuals with immune-dysfunction or immunosuppression (Kaiser et al., 2006).

The nasopharynx has been identified as the major site of focal virus production regardless of the inoculation route (Winther et al., 1986) and thus much of the data about specimen type and transmission route have immerged from investigations of URT infections. Nasopharyngeal washes result in better virus yield than either nasal or pharyngeal swabs (Cate et al., 1964; Peltola et al., 2008). HRVs retain their infectivity in the laboratory for hours to days on suitable surfaces indicating the practical possibility of self inoculation from the fomites (Hendley and Gwaltney, 1988). Thus frequent hand washing and disinfectant

wipes are very likely ways of interrupting virus transfer from fomites to the nose or to conjunctivae (Gwaltney and Hendley, 1978; Gwaltney et al., 1978; Hendley, 1998; Winther et al., 2007). However, as nose-picking and eye-rubbing occur frequently during the infection, inoculation can outpace the personal hygiene measures (Hendley et al., 1973).

Colds are the principal outcome ascribed to HRV infection but there is no obvious pattern of symptoms reported (Gern et al., 2000; Lidwell and Sommerville, 1951). Respiratory picornaviruses have been associated with more than 80% of common colds of which the majority are HRVs (Andrewes, 1966; Arruda et al., 1997). However, viruses like HRSV, HCoVs, IFVs and, human adenoviruses have also been detected in URT syndrome of illness (Andrewes, 1964, 1966; Eccles, 2007; Pizzichini et al., 1998). Generally symptoms which develop within days include sneezing, nasal discharge (rhinorrhoea), nasal congestion/blockage, sore or irritated throat, cough, headache, feeling of fever or chilliness and malaise (Rosenbaum et al., 1971; Winther et al., 2007). Loss of taste and smell, body aches and pains, hoarseness, mild burning of eyes and feeling of ear pressure due to obstruction/mucosal swelling may also occur. Moreover, experimentally infected adult volunteers have been reported to show anorexia, loose bowels and neutrophilia (Cate et al., 1965; Cate et al., 1964; Douglas et al., 1966). A detectable increase in body temperature is more likely seen in children than in adults but the fever can be found in either of the group (Andrewes, 1966; Dick et al., 1967; Miller et al., 2007). Although acute otitis media (AOM) was principally considered of bacterial aetiology, URT infection caused by bacteria is commonly accompanied by viral detection (Lehtinen et al., 2006). Indeed, HRVs have been strongly attributed to AOM (Vesa et al., 2001).

It is important to note that acute lower respiratory tract (LRT) infections cause more morbidity and mortality than HIV infection, malaria, cancer or heart attack (Mizgerd, 2006). Unfortunately, the significance of HRV infection in LRT morbidity during the first year of life is underappreciated (Kusel et al., 2006). Neonates and infants are more susceptible to poor outcomes from SRIs as a result of airway swelling, excessive secretions and smooth muscle contraction during ARTI (Bardin et al., 1992). Acute wheezing episodes (including bronchiolitis and acute asthma which share similar pathologies) are common manifestation of respiratory virus infection of the URT and LRT of children (Glezen et al., 1971; Henderson et

al., 1979; Rakes et al., 1999). Although the mechanisms underlying the induction or exacerbation of asthma are not yet fully understood (Bardin et al., 1992; Martinez, 2007) but excessive use of antibiotics is thought to be responsible for wheezing (Jartti et al., 2004; Mallia and Johnston, 2006; Pattemore et al., 1992). Exacerbations of asthma and COPD are usually preceded by a symptomatic rather than asymptomatic HRV episode although in some instances, an exacerbation is the only evidence of infection (Green et al., 2007; Heymann et al., 2005; Johnston et al., 1995; Lidwell and Sommerville, 1951; Minor et al., 1974; Pattemore et al., 1992; Roldaan and Masural, 1982). Respiratory picornaviruses are associated with reduced peak expiratory volume in children (Johnston et al., 1995). Bacterial infection was once considered to play a central role in expiratory wheezing but today it is well known that acute asthma exacerbations in all age groups are most often attributed to HRV infections (El-Sahly et al., 2000; Johnston et al., 1995; Silva et al., 2007). Asthma in children also seems to be a risk factor for more frequent symptomatic viral infections. However, the presence of allergy does not appear to be a common feature (Pattemore et al., 1992; Rakes et al., 1999) since only a small proportion of allergic children have asthma (Yoo et al., 2007). It is not clear if the risk of atopic asthma during infancy is augmented by SRIs (which affect the development of the immune system) or whether SRIs trigger asthma development in children with a genetic predisposition to more severe outcomes following infection by HRVs (Gern and Busse, 2002; Hershenson and Johnston, 2006; Martin et al., 2006).

Rhinovirus infections and immunology

HRVs are generally known as weapons of mass induction rather than destruction; in contrast to HRSV and Influenzavirus A (FLUAV), they target relatively few cells and impart little damage upon them. Thus it is the immune response to the HRV infection that is believed to drive illness (Dreschers et al., 2007; Hendley, 1998; Sung et al., 1993; Turner et al., 1998). *Ex vivo* studies of peripheral blood mononuclear cells from normal and asthmatic subjects pinpoint a shift towards a T helper cell-2 (T_H-2) response after HRV infection of cells obtained from the asthmatic individuals. The T_H-2 response manages humoral immunity and stimulates B cells via various interleukin (IL) like IL-4 (initiating production of IgE), IL-5 (influencing eosinophils) and IL-13 (a crucial component of allergen-induced asthma). The

T_H-1 response deals with cellular immunity producing IL-2 and IFN-γ. These two T cell responses act in concert with epithelial-derived chemokines to promote the recruitment and activation of eosinophils and mast cells, which contribute to chronic airway inflammation and the hyper-responsiveness of airways to a variety of non-specific stimuli (Gern and Busse, 2002). Although it is upregulated by HRV infections, the T_H-1 response is comparatively deficient in asthmatics (McFadden, 2003; Papadopoulos et al., 2002; Wark et al., 2005). It should be noted that an increased T_H-1-like cytokine response, deduced from higher sputum mRNA IFN-γ/IL-5 values, clears HRV and rapidly resolves the symptoms (Gern et al., 2000). Bronchial epithelial cells from asthmatic are also impaired in induction of the type III IFNs, IFN-λ1 and IFN-λ2/3, but in normal subjects, levels are inversely associated with HRV load and symptom severity (Contoli et al., 2006). HRV replication in bronchial cells induce the production of IFN-γ-induced protein 10 and RANTES; the former protein is a useful biomarker of virus-induced acute asthma associated with more severe airflow obstruction and reduced response to bronchodilators (Wark et al., 2007). HRV binding to ICAM-1 may alter neutrophil migration and T lymphocyte-mediated cytotoxic or T_H interactions with HRV-infected cells by upregulating receptor expression (Hakonarson et al., 1998). ICAM-I stimulation may also encourage eosinophil and T cell infiltration into the lower airways of asthmatic individuals and disrupt normal neutrophil trafficking potentiating the bacterial infections observed in AOM (Martin et al., 2006; Staunton et al., 1989a; Sung et al., 1993). Thus, higher viral loads and more inflammatory mediators are produced by cells from asthmatics than cells from non-asthmatics. One possible reason of the T_H-1 deficiency in asthmatics is inadequate maturation of type I and III IFN responses due to reduced exposure to infections in early life (Johnston, 2007). One intriguing theory is that HRV infections play a key role in developing an efficacious antiviral immunity, particularly in infancy, via their ubiquitous, frequent and usually mild, self-limiting infections (Yoo et al., 2007).

Older children and adults have greater amounts of HRV-neutralizing antibody than younger children, indicating the involvement of humoral immune response to HRV infections (Mogabgab and Pelon, 1957). Secreted anti-HRV antibody (mostly IgA) appears at about the same time as serum antibody (2 weeks after inoculation of healthy adult volunteers) and is retained at peak levels for at least 8 weeks (Cate et al., 1966; Cooney and Kenny, 1977). The IgA response does not seem to modify virus shedding or illness but protects against

reinfection. Other studies have identified that adults, without pre-existing nasal antibody to an experimental challenge virus, may succumb to more severe SRI and shed more virus for longer period (Buscho et al., 1972; Holmes et al., 1976). Antibody levels in nasal washes fall comparatively faster than serum levels, especially in isolated populations, which might explain why volunteers with pre-study serum antibody (but presumably no IgA) can still become infected (Buscho et al., 1972; Cate et al., 1965; Dick et al., 1967; Holmes et al., 1976). Notably, there is also evidence for some degree of nasal immune memory (Buscho et al., 1972).

Treatment of rhinovirus infections

Due to the large number of HRV types, it is practically impossible to develop a vaccine active against all serotypes. However different strategies have been used to deal with HRV infections. These strategies extend from interfering with the immune response to inhibit virus binding, entry, uncoating or protein processing. Acute infection is normally treated with 1st generation antihistamines to resolve cold symptoms leading to the reduction of sneezing and rhinorrhea. Although their application in therapy is limited due to side effect, non-steroidal anti-inflammatory drugs have proven to reduce fever and soreness of the throat, and might also have some positive effects on cough. Ipratropium bromide, an anticholinergic nasal spray, has been attributed to reduce rhinorrhea by 30% in natural cold and nasal blockage can be efficiently reduced with orally or intranasal administered decongestants. Some other nasal sprays like chromoglycate generally improve some respiratory illness including cough and voice disturbances. Treatments by dietary supplements such as, alcohol consumption, Echinacea and zinc have produced inconsistent benefits although their mechanism of action still remains elusive.

Apart from above drugs, certain chemicals have been designed to stabilize or inhibit the receptor binding to the virion capsid. Capsid-binding compounds block viral uncoating and/or viral attachment to host cell receptors. The three-dimensional structure of rhinoviruses determined by X-ray crystallography identified a hydrophobic pocket located beneath the canyon occupied by a pocket factor, a fatty acid or a similar hydrophobic compound with a polar head group derived from cellular components (Rossmann et al., 2002). This discovery

led to the development of a series of small hydrophobic, flexible, organic compounds that could replace the pocket factor and thereby increased the stability of the virus. The increased stability of the virus makes it more resistant to uncoating. Additionally, the binding of hydrophobic compounds into the pocket can induce structural changes in the canyon floor, resulting in an inhibition of receptor attachment (Rossmann et al., 2002). Thus, the attachment of major group HRVs to the cell surface receptor is inhibited whereas, minor group HRVs are inhibited due to the blocking of uncoating. However, compounds targeted against the capsid such as the "WIN" compounds (e.g. Disoxaril) or the "R"series of capsid binders (e.g. Pirodavir) failed in clinical trials because of vexatious side effects (Rotbart, 2002; Turner et al., 1993) or even the lack of clinical benefis at all (Hayden et al., 1995). Pleconaril was the first of a new generation metabolically stable capsid inhibitor that reduced the duration and severity of colds without any adverse side effects (Florea et al., 2003; Hayden et al., 2003). However, due to interference with the pharmacokinetic of other drugs it was rejected by the Food and Drug Administration (FDA). The discovery of ICAM-1 as the main cellular receptor for rhinoviruses led to the efforts to block the attachment of the virus to this receptor, using a soluble form of ICAM-1. However, such preparations (tremacamra) exhibited only modest effects in experimentally induced HRV infections (Turner et al., 1999). Another approach was the generation of multivalent recombinant antibody Fab fusion proteins directed against ICAM-1 (CFY196) but the efficacy of this protein still awaits proof in clinical trials (Fang and Yu, 2004).

Enviroxime, a drug which interferes with RNA replication, demonstrated potential antiviral activity against HRVs and enteroviruses *in vitro* by targeting the 3A function and thus inhibiting the initiation of plus-strand RNA synthesis (Heinz and Vance, 1995). Apart from high potency and a broad spectrum of action in cell culture, the clinical development was stopped because of intolerance to oral administration and limited antiviral activity after intranasal use (Anzueto and Niederman, 2003). Based on the same mode of action, a novel lead series of antipicornavirus drugs has been synthesized showing comparable antiviral activity to enviroxime *in vitro* however, there are no reports about clinical trials (Hamdouchi et al., 2003).

During the last few years, much of the focus has been centered on the 3C protease of picornaviruses as a potential target of antiviral therapeutics. Blocking the 3C protease activity

results in inhibition of virus specific polyprotein processing (Patick and Potts, 1998). The 3C coding region shows a significant degree of homology not only among HRV types but also among several related picornaviruses, and the absence of a known cellular homologue, makes it an attractive target for antiviral intervention. Rupintrivir (AG7088) is the most promising drug candidate today which is an irreversible 3C protease inhibitor and is currently tested in large-scale phase II/III clinical trials in patients with naturally acquired colds or in volunteers exposed to HRV infections (Witherell, 2000). New strategy of inhibition of viral replication mediated by small interfering RNAs (siRNAs) corresponding to the genome of HRVs has also been established (Phipps et al., 2004). Phipps and colleagues showed the ability of several short dsRNA molecules complementary to region of the HRV genome to be potent, specific inducers of RNA silencing in the cell, resulting in efficient inhibition of virus replication.

2. Materials and methods

Chemicals

All the chemicals used in this study were from sigma Aldrich otherwise stated.

Amiloride; was dissolved in PBS to make a 0.5 M solution. The resulting solution was divided in aliquots and stored at -20°C.

Bafilomycin A1; was purchased from ALEXIS Biochemicals, Switzerland.

Chlorpromazine; a stock solution of 10 mg/ml was prepared and stored at 4°C.

Cytochalasin D; was dissolved in DMSO at 1 mg/ml and stored at -20°C.

Dynasore; was dissolved in DMSO at 16 mM concentration and stored at -20°C.

Filipin; was dissolved in methanol at 10 mg/ml and stored at -20°C.

Heparin and heparan sulfate; porcine liver derived heparin and heparin sulfate were dissolved in PBS at 50 mg/ml and stored at either 4°C for short period or at -20°C for longer period.

Heparinase H; was dissolved in PBS and stored at -20°C.

Methyl-β-cyclodextrin (MβCD); 1 M solution was prepared in PBS and stored at 4°C.

Na-chlorate; was from Alfa Aesar GmbH, Germany.

Cells and Media

HeLa cells (Strain Ohio)

Human cervical epithelial tumor cell line was obtained from *European Collection of Cell Cultures (ECACC), Salisbury, Wiltshire, UK* and cultured in Minimal Essential Medium (MEM) with 1% L-glutamine, (*GIBCO BRL*) supplemented with 10% heat-inactivated fetal calf serum (FCS; *GIBCO BRL*) and 1% antibiotic (penicillin/streptomycin *GIBCO*).

Rhabdomyosarcoma (RD) and RD-ICAM cells

Human muscle fibroblasts wild type (RD) and stably transfected with human intercellular adhesion molecule-1 (RD-ICAM) were kindly gifted by Dr. Darren S. Shaffren *New Castle Australia* and propagated in Dulbico Modified Essential Medium (DMEM) supplemented with L-glutamine, FCS and antibiotics as above.

Chinese Hamster Ovary (CHO) cells

CHO-K1 (wild type) cells and mutants (pgs A-745 and pgs D-677) deficient in proteoglycan biosynthesis were obtained from the American Type Culture Collection (ATCC). These cells were grown in Ham's F-12 medium supplemented with 5% FCS, L-glutamine and antibiotics as above.

Culturing, Splitting and Seeding of the Cells

Cells stored in liquid nitrogen in freezing medium (10% DMSO in FCS) were quickly thawed and transferred to pre-warmed growth medium. Cells were centrifuged to get rid of DMSO and shifted to the flasks. After full confluency, cells were washed with PBS and splitted using trypsin-EDTA. A required number of cells were transferred to respective plates accordingly.

Viruses

All HRV types used in this study (HRV2, 8, 14, 18, 24, 54, 56, and 98) were originally from ATCC and were propagated in HeLa cells from a single isolated plaque. Identity of each serotype was ascertained using guinea pig antisera from ATCC via standard neutralization tests.

Seed virus production

HeLa (Ohio or H1) cells were grown in 10 large flasks up to 80% confluency. Cells were washed and infected with respective virus at multiplicity of infection (MOI) of 1 and incubated at 34°C till a clear CPE was evident. Cells were lysed by 3 freeze/thaw cycles and centrifuged for 20 min at 4300 rpm to remove the cell debris. The clear supernatant was

incubated overnight with 7% polyethylene glycol (PEG) 6000 plus 1.74% NaCl to concentrate the virus. Virus was pelleted along with PEG and collected in 10 ml of virus buffer A (VBA). PEG was removed by sonication for 3 min and centrifuged in SS-34 rotor for 30 min at 15000 rpm to obtain clear supernatant. Virus from supernatant was stored as 1 ml of aliquot each at -80°C for further use.

VBA: 20 mM Tris/HCl pH 7.5, 2 mM $MgCl_2$

Virus titer determination

Seed virus was serially diluted (1:10) and HeLa cells grown in 96 well plates up to 40-50% confluency were infected with each dilution. Plates were incubated at 34°C for 5 days and stained with 0.1% crystal violet to assess the cell death. Virus titer in terms of 50% tissue culture infection dose ($TCID_{50}$/ml) was calculated according to Reed's method (1938).

Neutralization test

Identity of each virus serotype was monitored via neutralization test. Briefly serotype specific guinea pig antiserum was diluted 1:2. Seed virus at 1000 $TCID_{50}$/well of a 96 well plate was mixed with each dilution and incubated at 34°C for 1.5 h to allow the antibody to react with the virus particles. The mixture was transferred to 50% confluenct HeLa cells grown in 96 well plates. Plates were incubated at 34°C for 3-5 days and were stained with crystal violet to reveal the cell death or protection due to neutralizing ability of the antibody used for specific rhinovirus type.

Plaque purified virus preparation

To obtain homogenous virus population, seed virus was prepared from a single plaque picked through three successive plaque picking processes. Generally HeLa or RD cells were grown in 6 well plates and virus dilutions at 10^1 to 10^5 $TCID_{50}$ were adsorbed on the sub-confluent

cells for 1.5 h at 34°C. Unbound virus was removed and cells were over laid with 3 ml of 1% low melting agarose in MEM. After 3 days at 34°C, cells were stained with neutral red. Plaques were marked and carefully picked and transferred to 1 ml infection medium. A random plaque was further boosted and processed through the same procedure to grow further plaques. In this way, a plaque after third round was chosen to produce seed virus.

Purified virus production

HeLa cells grown in 10 large flasks (162 cm^2) to 80% confluency and were infected with virus at MOI of 1. After 80-90% CPE, cells were broken by three freeze/thaw cycles and centrifuged for 30 min at 20000 rpm, 4°C in SS-34 rotor to remove cell debris. Supernatants were transferred to Ti-45 tubes while cell pellet was dissolved in virus buffer B (VBB) + 4 M NaCl, sheered in douncer for 40 times and sonicated for 3 min. The mixture was again centrifuged in SS-34 rotor to get rid of debris. Supernatants were transferred to Ti-45 tubes containing previous virus supernatants. The tube was filled with VBB + 4 M NaCl and centrifuged to pellet the virus at 30000 for 2.5 h. The pellet was resuspended in 200 µl of VBA + 4 M NaCl containing 10 µl of each DNase and RNaseA (5 mg/ml) and incubated for 10 min at room temperature. Hundred µl of trypsin-EDTA was added and mixture was incubated at 37°C for 5 min. Twenty µl of laurylsarcosyl was added and incubated overnight at 4°C. The mixture was mixed gently and centrifuged in eppendorf tube for 15 min. The clear supernatants were applied on top of 7.5-45% sucrose gradient prepared in VBA + 4 M NaCl. The pellet was resuspended in VBA + 4 M NaCl, sonicated for 15 min and centrifuged again in table top eppendorf centrifuge for 15 min. The clear supernatants were added to the top of gradient. The gradient was centrifuged for 2 h, 35000 rpm in SW-40 rotor. Fractions of 1 ml each were collected. Each fraction was tested for the presence of virus via SDS-PAG electrophoresis. Briefly 80 µl from each fraction was mixed with 20 µl TCA, incubated on ice for 15 min and centrifuged for 5 min at 14000 rpm. Pellete was washed twice with 70% ethanol and dried in speed-Vac. Twenty µl of VBA was added and sample was loaded on 15% SDS-PAGE. Proteins were detected with coomassie staining. Fractions containing pure virus were mixed and put into SW-40 tubes. Tubes were filled up to 10 ml with VBA and

centrifuged at 35000 rpm overnight. Virus pellet was resuspended in HEPES buffer and re-checked through SDS-PAGE for virus purity.

VBB: 10 mM Tris/HCl, 10 mM EDTA pH 7.4

HEPES buffer: 140 mM KCl, 10 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanosulfonoacid) pH 7.4

Preparation of [^{35}S]-labeled virus

HeLa cells grown in 162 cm^2 flasks to about 80% confluency, were washed with PBS, and incubated with 20 ml methionine/cysteine free MEM supplemented with 2% dialysed FCS, antibiotics, L-glutamine, and 30 mM MgCl$_2$ for 4 h at 37°C. Cells were challenged with seed virus at MOI of 100-500 in fresh methionine/cysteine free MEM as above. After 4 h of incubation at 34°C to allow virus internalization and host cell shut off, 15 ml of the old medium was replaced by fresh medium containing 1mCi [^{35}S]-methionine (Hartmann Analytic GmbH, Braunschweig, Germany). Incubation was continued for further 16 h when a complete cytopathic effect (CPE) was evident. After 3 cycles of freeze/thaw, cell debris was removed by centrifugation at 20000 rpm (Ty65 rotor) for 20 minutes at 4°C. The clear supernatant was subjected to another centrifugation step at 30.000 rpm (Ty65 rotor) for 80 minutes at 4°C to pellet the virus. Virus pellet was then resuspended carefully in 200 μl VBA and incubated over night at 4°C. Eventually unsolubilized material was removed by centrifugation in an Eppendorf table top centrifuge. A continuous density gradient ranging from 10-45% sucrose in VBA was prepared in a SW40 centrifuge tube and the virus suspension was transferred on top of the gradient and centrifuged at 35,000 rpm (SW40 rotor) for two h at 4°C. Fractions of 300 μl each were collected from the bottom of the gradient by means of a needle and a pump and were analysed for incorporated radioactivity by liquid scintillation counter. In order to examine the purity of the virus preparation, aliquots of each fraction were run on a 15% SDS-PAGE under reducing conditions. The gel was dried and exposed to a Kodak BioMax MR film for overnight or scanned with phospho-imager to detect viral capsid proteins.

Preparation of [^{35}S]-labeled virus in 6 well-plates

For smaller scale virus production, HeLa cells were grown in a 6 well plate to about 80% confluency. The medium was aspirated and cells were washed with PBS. Cells were challenged with virus at 10^8 TCID$_{50}$/well in 300 μl PBS at 34°C with gentle rocking. After 1 h of incubation, 500 μl of methionine/cysteine free infection medium was added and the incubation was continued for further 3 h at 34°C. Cells were washed to remove unbound virus. 800 μl of fresh methione/cysteine free infection medium was added and after 3 h of incubation, 0.1mCi/well of [^{35}S]-methionine was added. After approximately >80% CPE, cells were broken by three freeze/thaw cycles and cell debris was removed by low speed centrifugation for 30 min at 4°C. The supernatants containing the virus were centrifuged at 70,000 rpm in a table top ultracentrifuge (rotor TLA 100.3, Beckman Instruments, USA) for 1 h at 4°C. The supernatants were discarded and the pellet was resuspended in 50 μl of VBA for overnight. After a complete resuspension, virus suspension was filled up to 1 ml with VBA and re-pelleted to get rid of free methionine. Virus pellet was resuspended in 50 μl of VBA. Viral proteins in the preparation were separated on a 15% SDS-PAGE. The gel was dried and either scanned using a phosphor imager or exposed to Kodak film as above.

Expression and purification of VLDLR fusion proteins

A starting culture inoculated from a glycerol stock containing the appropriate plasmid was grown in 50 ml LB-amp medium at 37°C. The O/N culture was diluted 1:100 in 2 L LB-amp medium and grown to an OD$_{600}$= 0.7 at 37°C. Protein expression was induced by adding 0.3 mM IPTG and the bacterial culture was incubated for another 20 h at 30°C to optimize fusion protein yield. After centrifugation for 20 min at 5000 rpm in rotor GS3, the bacterial pellet was washed once with 50 ml TBSC and centrifuged again to obtain a clear pellet. The final bacterial pellet was resuspended in TBSC in a total volume of 30 ml and transferred into 50 ml Falcon tubes at 4°C. The bacterial culture was ultrasonicated 6 times for 10 seconds on ice with 30 sec delay between each cycle to prevent overheating of the protein solution. The solution was centrifuged for 10 min at 4300 rpm in a Megafuge 1.0 and the supernatant was transferred into SS-34 tubes for a second centrifugation of 20 min at 14000 rpm, 4°C to pellet

the cell debris (rotor SS-34). The supernatant was kept for further purification. The protease inhibitor PMSF (2 mg/ml stock solution) was added to inhibit protein cleavage by cellular proteases. The supernatant was incubated at 4°C rotating with at least 5 ml Ni-NTA slurry in 50 ml falcon tubes over night to bind the proteins via His-tags on the beads. The beads were centrifuged for 20 min at 2500 rpm at 4°C (Heraeus SEPATECH Megafuge 1.0R). An aliquot of the supernatant was loaded onto a 15% SDS-PAGE to ensure that the proteins were bound by the beads and nothing was left in the supernatant. The beads with the bound proteins were washed 3 times with 1XTBSC + 10 mM Imidazol by rotating for 5 min and centrifugation for 10 min at 4°C. 4 ml TBSC + 250 mM Imidazol was added and the slurry was incubated for 1 h at 4°C to elute the proteins and centrifuged for 15 min at 2500 rpm. This step was repeated 5 times with 20 min incubation at 4°C rotating to obtain a total eluation volume of 20 ml. An aliquot of the eluted proteins (5 μl) was analysed by 15 % SDS-PAGE and coomassie staining to examine protein purity. The protein concentration was determined by spectrophotometry at 280 nm.

TBSC: 25 mM Tris (pH 7.5), 150 mM NaCl, 2 mM $CaCl_2$

Membrane proteins extraction

Cells were grown in 2X 154 cm^2 dishes up to full confluency, washed with PBS and detached with cell scraper. Cells were collected in 15 ml falcon in PBS and pelleted in Magafuge 1.0 for 5 min at 1200 rpm. Pellet was washed once with PBS and resuspended in 1 ml lysis buffer on ice. Cells were broken by three freeze/thaw cycles and ultracentrifuged at 70,000 rpm in TLA 100.3 rotor for 40 min at 4°C. Supernatats were discarded and pellet was resuspended in 100 μl detergent buffer and kept on ice for 10 min. Cell debris was removed by another step of ultracentrifugation as above. Membrane proteins in the supernatant were stored at -20°C in 10 μl of each aliquote.

<u>**Lysis Buffer**</u>

200 mM Tris-maleate pH 6.5 (2.42g Tris and 2.32g maleate to 100ml), 2 mM $CaCl_2$; 0.5 mM, PMSF (it is unstable in aqueous solution, so lysis buffer was prepared just before use), 2.5 μM Leupeptin

<u>**Detergent Buffer**</u>

Lysis buffer + 1% triton X-100

Virus overlay blot (VOB)

Whole cell membrane extracts or concatemers of VLDLR (V3X5 and VI-8) were loaded on 6 and 12% SDS-PAGE respectively. Proteins were transferred to a PVDF membrane via semidry transblot for 1.5 h. The membrane was blocked in 5% non-fat milk dissolved in TBSC for 1 h at room temperature or overnight at 4°C. [^{35}S]-labeled virus at ~100,000 cpm in 5 ml TBSC was added to the membrane and incubated for 1 h at RT or overnight at 4°C. Unbound virus was removed by 3X washings with TBSC. The membrane was air dried and exposed to Kodak BioMax MR film for overnight or scanned via phosphor-imager to detect virus interaction with the proteins.

Infection of RD cells by K-type viruses

Cells were grown in 48 well plates up to 70% confluency and were infected with seed viruses in infection medium at 100 $TCID_{50}$/cell. After 24 h of incubation at 34°C, cells were either stained with crystal violet to assess CPE or analysed using cell viability assay (Promega celltiter kit).

Cell infection inhibition assays

RD cells were grown in 48 well plates and infected with HRV54 at 50 $TCID_{50}$/cell in the presence or absence of 50 μg V3X5, 2 mg/ml heparin and heparin sulfate (HS). HRV2 was used as a control (for V3X5). Plate was incubated at 34°C, 5% CO_2 until cells in the control

showed more than 90% CPE (36 h generally) and were stained with crystal violet to assess cell death protection in the presence of inhibitors. For quantitative analysis, cells were grown in 96 well microtiter plates up to 80% confluency and infected in the presence or absence of serial dilutions of soluble heparin and HS in infection medium. Cells were fixed with 4% paraformaldehyde (PFA). After washing with PBS, cells were stained with 0.1% crystal violet. Plate was washed with water and stain was eluted with 150 µl of absolute methanol. Intensity of the stain was quantified taking O.D at 630 nm in a microtiter plate reader.

Double receptor specificity assays

RD-ICAM cells were grown in 96 well plates and infected by HRV54 at 10 $TCID_{50}$/cell in the presence or absence of R6.5 monoclonal antibody (anti-ICAM-1) and heparin alone or in combinations. Cells were incubated for 1 h in 100 µg/ml of R6.5 before the addition of virus. On the other hand, virus was incubated with 2 mg/ml of heparin for 1 h at 34°C prior to cell infection. After 24 h of incubation at 34°C, the cells were fixed, stained and eluted with methanol as described above. O.D was taken at 630 nm.

Effect of BafilomycinA1 on virus uncoating

RD cells grown in 96 well plates were incubated with 200 nM of Bafilomycin A1 for 1 h at 37°C. Virus at 100 $TCID_{50}$/cell along with 100 nM of BafilomycinA1 in infection medium was added to the cells and incubation was further continued for 24 h at 34°C. To investigate the role of ICAM-1 in virus uncoating, RD-ICAM cells were used in parallel. However in addition to bafilomycin A1, cells were also incubated with 100 µg/ml of R6.5 for 1 h to block ICAM-1 prior to virus addition. Infectivity was evaluated by taking the O.D as above.

To test whether viral *de novo* synthesis occurs in the presence of Bafilomycin A1, we also carried out virus kinetics assays. RD and RD-ICAM cells were grown in 24 well plates up to 80% confluency. Cells were washed with PBS and incubated with or without 200 nM bafilomycin A1 in 200 µl of infection medium for 1 h at 37°C. Virus at 10^6 $TCID_{50}$ in 200 µl of infection medium was added and incubation was continued for another hour at 34°C. Cells

were washed twice with ice cold PBS and 200 µl of infection medium with or without 100 nM bafilomycin A1 was added. Incubation was continued for further 0, 2, 12 and 23 hours. After indicated incubation periods, cells were lysed by three freeze/thaw cycles. Cell debris was pelleted and virus titer in the supernatant was determined by end point dilution test.

Acid sensitivity of the virus

Virus at 10^7 TCID$_{50}$ was incubated with 0.5 M sodium acetate buffer of pH 7, 6.5, 6, 5.2, 4.8 and 4.4 for 30 min at room temperature and neutralized by 0.5 M sodium phosphate buffer. A serial dilution from each fraction was made and the infectivity of the virus was determined by an end point dilution assay.

Cell binding assays

RD cells were grown in 12 well plates, washed with HBSS (Hank's Buffered Salt Solution). [^{35}S]-labeled virus at ~12000 cmp in HBSS was incubated in the presence or absence of inhibitors for 60 min at 34°C by gentle rocking. Cells were washed to remove unbound virus and were detached with trypsin-EDTA treatment. Cell associated radioactivity was determined by scintillation counter.

HBSS: 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na$_2$HPO$_4$, 0.44 mM KH$_2$PO$_4$, 1.3 mM CaCl$_2$, 1.0 mM MgSO$_4$, 4.2 mM NaHCO$_3$

Enzymatic digestion of cell surface heparan sulfate

RD cells grown in 24 well plates up to 80% confluency were washed with PBS and incubated with 4, 2 and 1 U/ml of Heparinase1 in HBSS for 2 h at 37°C. Cells were washed 3X with ice cold buffer to remove digested material and [^{35}S]-labeled virus at 16000 cpm was added and incubation was continued for another hour at 4°C. Cells were washed and trypsinized. Cell associated radioactivity was determined by scintillation counter.

Antibodies and reagents

Mouse anti-HRV8, mouse anti-HRV14, rabbit anti-HRV14, and monoclonal mouse anti-HRV2 (8F5) (Hewat and Blaas, 1996) were produced following standard protocols. Goat anti-clathrin heavy chain, rabbit anti-caveolin, and rabbit anti-flotillin-1 antibodies were purchased from Santa Cruz Biotechnology. Secondary antibodies labeled with Texas red, Alexa Fluor 488, and Alexa Fluor 591 were from Jackson Antibodies and Molecular Probes, respectively. Alexa Fluor 568 transferrin, Alexa Fluor 488 cholera toxin-B, and lysine-fixable FITC-dextran were purchased from Molecular Probes, Invitrogen.

Drug treatment

The following drugs were used to inhibit a particular pathway(s); chlorpromazine in PBS was used at 5 µg/ml. Filipin dissolved in methanol was used at 10 µg/ml, MßCD dissolved in PBS was used at 5 mM. Amiloride, cytochalasin D, and dynasore were dissolved in DMSO and used at 5 mM, 10 µg/ml, 80 µM, respectively. Briefly RD and RD-ICAM cells were grown in 24 well plates. Two parallel sets were pre-incubated in the presence or absence of the respective drug for 30 min and infected with HRV2, HRV8v, or HRV14 at 300 $TCID_{50}$/cell. After additional incubation for 30 min at 34°C, one set of samples was fixed and prepared for indirect fluorescent microscopy. The second set was harvested in 50 µl sample buffer at 3 h p.i. for assessment of eIF4G1 cleavage via western blotting. Drugs were present throughout the incubation.

Western blot analysis

Proteins from whole cell lysates from each drug treatment were separated on a 6% SDS-polyacrylamide gel and electro-transferred onto a PVDF membrane. The membrane was blocked with 5% milk powder in PBS/T (PBS + 0.1% tween-20), incubated with rabbit anti-eIF4G1 (1:8,000) kindly donated by R. Rhoads, Louisiana State University, USA, followed by HRP-labeled anti-rabbit antibody (1:20,000; Jackson Laboratories) and, eIF4G1 and its

cleavage products, were visualized by using the ECL chemiluminescence detection system (Thermoscientific).

Confocal fluorescence microscopy

Cells were grown on glass cover slips until 40-50% confluent. After washing with serum-free medium, cells were challenged with HRV2, HRV8v, or HRV14 at 300 or 500 $TCID_{50}$ /cell as specified. Unbound virus was removed by three washings with PBS++ (PBS with 1 mM $MgCl_2$ and 1 mM $CaCl_2$). Cells were fixed in 4% PFA, and permeabilized in 0.2% Triton-X 100 for 5 min. After quenching with 50 mM NH_4Cl for 10 min, cells were blocked in 1% goat serum for either 1 h at room temperature or overnight at 4°C. Cells were incubated for 1 h at room temperature with primary antibody (1:500), washed 3 times with PBS++, followed by secondary antibody (1:400), all diluted in 1% goat serum. Nuclei were stained with 0.1 µg/ml of DAPI. For assessment of colocalization, cells were infected in the presence of 10 µg/ml Alexa Fluor 568 transferrin, 1 µg/ml Alexa Fluor 488 CtxB, and 500 µg/ml lysine-fixable fluorescein-dextran (70 kD), respectively. After the times specified, unbound material was removed and cells were fixed. Virus was detected by using type-specific antibodies as above. Cover slips were mounted in Moviol and viewed in a LSM510 Meta Carl Zeiss confocal microscope. Stacks were spaced by 0.5 µm where applied.

Transfection

The plasmids pEGFP-C3, encoding the clathrin light chain fused to green fluorescent protein (GFP-Clathrin), myc-tagged pCMV-Amph-SH3, and pMCV-AP180 were kind gifts from Luc Snyers. They have been used and described previously (Snyers et al., 2003). Cells were seeded on cover slips in 24 well plates and grown to 30% confluency. One µg plasmid DNA was mixed with 50 µl Optimem (Gibco). Separately, 3 µl Lipofectamine 2000 (Invitrogen) was mixed with 50 µl Optimem and incubated for 5 min at room temperature; the 2 solutions were mixed gently and incubated for 20 min at room temperature. Cells were washed twice with antibiotic-free growth medium and laid down with 500 µl of the same medium. The mixture of plasmid DNA and lipofectamine 2000 was applied onto the cells drop by drop with

gentle rotation to avoid cell toxicity. Cells were incubated for 3 h with the mixture; then, the medium was replaced with fresh antibiotic-free growth medium. Forty-eight hours post transfection, cells were infected with the respective virus and processed for immunofluorescence microscopy. For Amphi-SH3 and AP-180C anti-myc antibody was used to verify expression.

Electron microscopy

RD and RD-ICAM cells were seeded on aclar cover-slips in 24 well plates and grown to almost 100% confluency. Cells were washed and infected with HRV2, HRV8v, and HRV14, respectively, at 10,000 $TCID_{50}$ /cell. After incubation at 34°C for the given times, unbound virus was removed and cells were fixed with 2% freshly prepared glutaraldehyde for 1 h at room temperature. Cells were washed 3 times with 0.1 M Sorensen buffer (pH 7.3) and incubated for 1 h in 2% osmium tetroxide in the same buffer. After washing, cells were dehydrated with ascending concentrations of ethanol, 40%-60%-80%-95% and 100% (kept over a molecular sieve). Cells were incubated with Epon/100% EtOH (1:1) for 30 min and twice with Epon only, embedded in resin filled beam capsule lids, and incubated at 60°C to polymerize for 2 days. Sections of 70 nm were prepared, stained with 2% uranyl acetate and lead citrate for 10 and 5 min respectively, and viewed under an FEI Morgagni 100 kV electron microscope equipped with an 11 megapixel CCD camera.

Objectives

The most surprising result of studies focused on the early events of animal virus infections is the diversity of cell surface receptors. There is no obvious reason why one virus species would use receptor A while its very close relative may have selected a totally different molecule B or two entirely different viruses use the same receptor. Human rhinoviruses, the major cause of upper and lower respiratory infections, bind to two entirely structurally and functionally different receptors. The basis of receptor discrimination is still a major open question. Major group HRVs possess ICAM-1 recognition signature which are absent in the minor group viruses. On the other hand, a single lysine is strictly conserved in the HI loop of all minor group HRVs and is necessary for interaction with LDL receptors. Exceptionally, there are 9 major group HRVs that also have the lysine at identical position and are called K-type viruses. Using ICAM-1 blocking antibody, it was shown that the K-type viruses cannot use LDLR for productive infection. However, as the major group HRVs not only require ICAM-1 for binding but also for uncoating, we wondered that the K-type HRVs might bind and enter the cells but due to lack of uncoating failed to infect. To address this question, experiments were carried out to investigate virus-receptor interaction *in vitro* and *in vivo*. Furthermore, pH sensitivity and infection of ICAM-1 negative cells by K-type viruses was carried out.

After binding to respective receptors, viruses need to enter the cell and release the genome near/at suitable site for replication. The receptors for minor group HRVs carry clathrin clustering signal and hence, virus entry has been shown as clathrin-mediated endocytosis. On the other hand, major group HRVs and viruses that bind to HS, their entry mechanism is poorly understood. ICAM-1 lacks the clathrin clustering signal and still acts as a functional receptor when its cytoplasmic tail is replaced by a GPI anchor. Entry via HS has produced ambiguous data and there is no clear evidence for the involvement of a particular pathway. Thus, in second part of this study, we have investigated the entry mechanism of rhinoviruses when they bind to different receptors and finding of this study could lead to the development of novel drugs targeting the virus entry.

3. Results

Part-1 (Receptor Specificity of Human Rhinoviruses)

Interaction of K-type HRVs with LDLR and concatemers of VLDLR

Sequence comparison of VP1, the major exposed protein of the virion surface, revealed particular clusters of amino acid residues in the ICAM-1 binding site that are absent in representatives of the minor group; however, no such receptor recognition "signature" exists in minor group HRVs (Vlasak et al., 2003). Nevertheless, a single lysine is conserved in the HI loop of all minor group viruses that plays a major role in binding to LDL receptors. Exceptionally, there are also 9 major group HRVs called "K-type" that also possess a lysine at the same position; this suggested that they might be able to use LDLR for infection as well. However, all of these K-type viruses failed to infect HeLa cells in the presence of the ICAM-1 blocking monoclonal antibody R6.5 (Vlasak et al., 2003). As major group HRVs not only differ in receptor specificity but also in their pH sensitivity, most of them being more stable than representatives of the minor group, we assumed that these viruses could bind LDLR but due to high stability at low endosomal pH, they would be unable to uncoat. In order to address this question, we carried out virus overlay blot (VOB) assays to investigate the interaction of K-type viruses with V33333 (a derivative of VLDLR and a potent binder of HRV2). MBP fused soluble protein V33333 (V3X5) was loaded on 12% SDS-PAGE under non-reducing conditions and transferred to PVDF membrane. The membrane was blocked and incubated with [^{35}S]-labeled HRV8, 18, 24, 54, and 85 at ~100,000 cpm in TBSC. HRV2 and 14 were used as positive and negative controls respectively. After 1 h of incubation, the membrane was washed three times with TBSC. After drying, it was exposed to Kodak X-ray film overnight. To our surprise, HRV8, 18, and 24 showed significant binding to V3X5; however, this interaction was much weaker than HRV2 (Fig. 3.1A). Notably other K-type HRVs like 54 and 85 did not show any binding to this concatemer at all. The specificity of the interaction was confirmed by using EDTA and MBP where the former completely abolished binding and the latter alone did not show any interaction with the viruses (data not shown). However,

interaction between these K-type viruses and the receptor was below the detection limit when using co-sedimentation and FCS. Most probably in VOB, the high sensitivity of the detection was due to very high local concentration of the receptor on the membrane after electrotransfer.

LDLR has 7 ligands binding modules whereas VLDLR contains 8 such binding repeats. Apart from the preference of natural ligands for different repeats, it has been shown that different modules have different affinity for HRV2 (Nizet et al., 2005). Taking these differences into account, we decided to test the complete VLDLR protein with its 8 repeats to see whether the K-type HRVs might have some repeat preferences and would bind more strongly to the receptor containing all repeats. However, the VOB with VLDLR1-8 did not show any significant difference from that using V3X5 (compare Fig. 3.1A and 3.1B). Moreover, in accordance with previous data, only HRV8 and 18 showed binding whereas other K-type viruses failed to interact with the receptor (not shown).

Figure 3.1: Some of the K-type viruses bind to VLDLR concatemer. A) MBP fused to V3X5 or B) V1-8 were separated by 12% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked using 5% non-fat milk in TBSC and incubated with [^{35}S] -labeled HRV8, 18, 24, 54, and 85 at 100,000 cpm for 1h at room temperature. The membrane was washed three times with TBSC buffer and an image was acquired with the phosphor-imager for 1 h. HRV2 and HRV14 were used as positive and negative controls respectively.

Having observed binding of HRV8, 18 and 24 to the VLDLR concatemers, we carried out the VOB using whole cell membrane extracts to investigate the interaction with natural receptors. The membrane proteins prepared from HeLa cells were separated by 6% SDS-PAGE and

transferred to a PVDF membrane. Incubation of the membrane with [^{35}S]-labeled HRV2, 8 and 18 revealed binding neither of HRV8 nor of HRV18 whereas HRV2, used as a control showed significant binding to LDLR (Fig. 3.2). Notably, there was no virus binding to the LRP either (not shown). These results indicate and further strengthen the previous finding that K-type viruses that showed interaction with V3X5 exhibit much weaker affinity when compared to minor group HRVs and thus are unable to bind to LDLR.
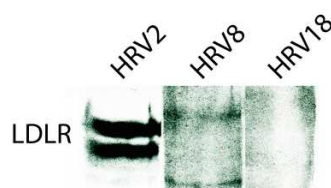

Figure 3.2: HRV8 and HRV18 failed to bind to LDLR. Membrane proteins extracted from the HeLa cells as detailed in Material and Method were separated by 6% SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked by using 5% non-fat milk powder in TBSC and incubated with [^{35}S]-labeled HRV8 and HRV18 at 100,000 cpm for 1 h at room temperature. The membrane was washed three times with TBSC buffer and image was acquired in the phosphor-imager for 1 h. HRV2 was used as a positive control.

Infection of Rhabdomyosarcoma (RD) cells with K-type viruses

After having noticed that some of the K-type HRVs can bind to VLDLR concatemers, we screened them for infection of ICAM-1 negative RD cells. Cells were grown in 48 well plates up to 70% confluency and infected with HRV8, 18, 24, 54, 56, 85 and 98 at 50 TCID$_{50}$/cell in infection medium. HRV2 and 14 were taken as positive and negative controls respectively as above. Thirty-six hours p.i., the cells were stained with crystal violet to assess the CPE. To our surprise, HRV54 caused a substantial cell death after 36 h of incubation (Fig. 3.3) even though it did not show any interaction with V3X5 on virus overlay blot assay while the other K-type viruses did not infect RD cells. However, as expected RD-ICAM (stably transfected with ICAM-1) cells were efficiently infected by all the viruses used in this assay.
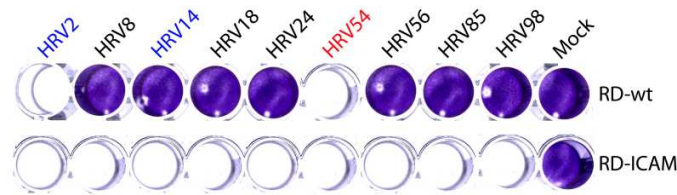
Figure 3.3: HRV54 infects ICAM-1 negative RD cells. RD (wt) and RD-ICAM cells were grown in 48 well plates up to 70% confluency and infected with each virus at 50 $TCID_{50}$/cell in infection medium. Plates were incubated at 34°C for 36 h. Medium was removed and cells were stained with 0.1% crystal violet to assess cell death. HRV2 and 14 were used as controls.

HRV54 infection is not inhibited by V3X5

Although HRV54 did not show any binding to minor group receptors in the VOB assays, we still wanted to assess whether the infection of RD cells was due to the involvement of minor group receptors or not. To this end, we infected the cells in the presence of V3X5 and monitored the CPE. As expected, HRV54 infection of RD cells was not inhibited by the presence of this potent inhibitor of minor group HRVs as demonstrated by HRV2 in this experiment (Fig. 3.4A).

This finding prompted us to look for other possible candidate receptors. Recently, several representatives of the *picornaviridae* family have been shown to use heparan sulfate proteoglycan (HSPG) as an alternative receptor. Moreover, HRV89 has been adapted to grow in cells devoid of ICAM-1 and these variants also use HSPG as a receptor (Vlasak et al., 2005a). Thus, after having noticed the ability of HRV54 to infect ICAM-1 negative RD cells and lack of inhibition by the VLDLR derivative V3X5, we wondered whether this serotype also used HSPG for cell attachment. Using the same approach as employed for V3X5, we infected the cells in the presence and absence of 2 mg/ml of soluble heparin (H) and heparan sulfate (HS). Interestingly, both inhibitors completely blocked the infection of RD cells by HRV54 (Fig. 3.4B). This result strongly suggested the involvement of cell surface HSPG in HRV54 infection of RD cells.
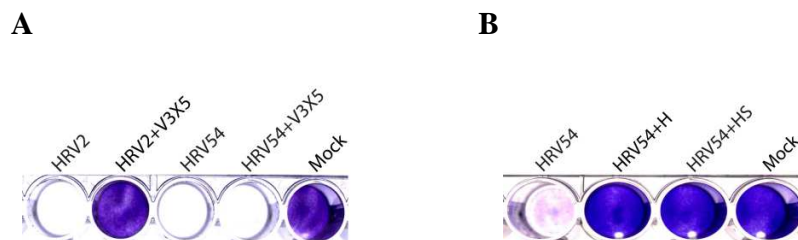
Figure 3.4: HRV54 infection of RD cells is inhibited by H and HS but not with V3X5. A) RD cells were grown in 48 well plates up to 70-80% confluency and were infected with HRV54 virus at 50 $TCID_{50}$/cell in infection medium in the presence and absence of 30 μg MBP-V3X5. HRV2 was used as a control. B) Cells were infected in the presence and absence of 2 mg/ml of soluble H and HS. Plates were incubated at 34°C for 36 h. Medium was removed and cells were stained with 0.1% crystal violet to assess the CPE.

In order to test the inhibition capacity of heparin and HS more precisely and quantitatively, we challenged the cells with HRV54 in the presence of two-fold serial dilutions of heparin and HS and cell survival in the presence of inhibitors was evaluated by relating it to 0% (in the absence of any inhibitor) and 100% (to the mock infected cells). As shown in Fig. 3.5, heparin and HS showed a concentration dependent inhibition of infection. Heparin, which is strongly sulfated, showed more inhibition than HS. Heparin at 1 mg/ml was sufficient to completely abolish CPE and a gradual decrease of inhibition at lower concentrations was evident. For HS, complete inhibition was observed at 2 mg/ml with almost no inhibition when present below 0.5 mg/ml.
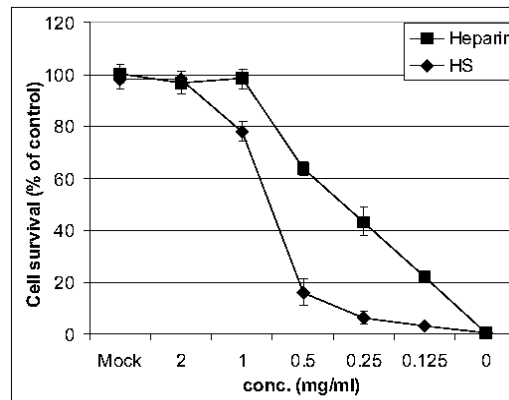
Figure 3.5: Heparin and HS inhibit CPE upon infection of RD cells with HRV54 in a concentration-dependent manner. RD cells grown in 96 well plates were infected with HRV54 (50 $TCID_{50}$/cell) in the presence or absence of the GAGs at the concentrations indicated. Plates were incubated at 34°C until cells in the control wells showed > 90% CPE. Surviving cells still sticking to the plastic were fixed, stained with crystal violet and the stain was eluted with methanol. The degree of CPE was quantified in a plate reader at lambda 630 nm. Cell survival in mock-infected cells (mock) was set to 100% and in the absence of GAGs to 0%. Results are the mean from three parallel experiments ± SD.

To exclude any role of heparin other than competing with the natural receptor, we carried out a cell protection experiment in three different ways (Escribano-Romero et al., 2004). 1) Virus was incubated with the heparin for 30 min and the mixture was then added to the cells; 2) Cells were incubated with virus at 4°C for 30 min and unbound virus was removed by washing prior to addition of heparin; and 3) Cells were incubated with heparin for 30 min and then was washed away before virus addition. As expected, we observed maximum inhibition of infection when the virus was incubated with heparin prior to challenging the cells. We also observed a protection of up to 40% when heparin was added after virus binding at 4°C but only at the highest concentration (1 mg/ml). This might be due to stronger interaction of heparin to already bound virus which could detach from the natural receptor. Finally, incubation of the cells with heparin did not modify the infection in any significant way (Fig. 3.6). These results clearly show that the soluble analogue of the cell surface proteoglycan physically interacts with the virus and thus blocks its binding sites for the cell surface receptors.
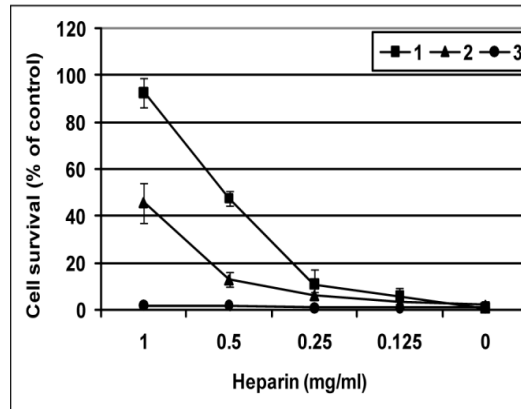
Figure 3.6: Heparin inhibits HRV54 infection of RD cells by interfering with virus binding. Inhibition of infection was assessed through three different ways. Virus was incubated with heparin for 30 min at 34°C and the mixture was then added to a monolayer of RD cells (1), cells were incubated with virus for 30 min at 34°C, washed to remove unbound virus and then heparin was added (2) and cells were incubated with heparin for 30 min and washed prior to virus addition (3). HRV54 was used at 50 $TCID_{50}$/cell. Cells were fixed, stained, washed, and stain was eluted with methanol. Color intensity was quantified in a plate reader. Percentage cell survival in the presence of heparin was evaluated as above. Mean of three parallel experiments ± SD.

Heparin and heparan sulfate suppress HRV54 binding to the cell

To further strengthen these results, we carried out binding inhibition assays using [^{35}S]-labeled virus. RD-cells grown in 24 well plates were washed with HBSS and incubated with radio-labeled virus at 16,000 cpm in the presence or absence of soluble GAGs at 34°C for 60 min with gentle rocking. Unbound virus was removed and cell-associated radioactivity was determined. As evident from Fig. 3.7A, heparin showed a concentration dependent inhibition of binding. We also tested other proteoglycans like HS, chondroitin sulfate (CS) and dermatan sulfate (DS) for their ability to inhibit virus binding. In accordance to the previous data only heparin and HS suppressed binding to background values while DS showed some inhibition and the effect of CS was only marginal (Fig. 3.7B). These results indicate that the virus uses preferentially cell surface HSPG for binding to cells that are devoid of ICAM-1.
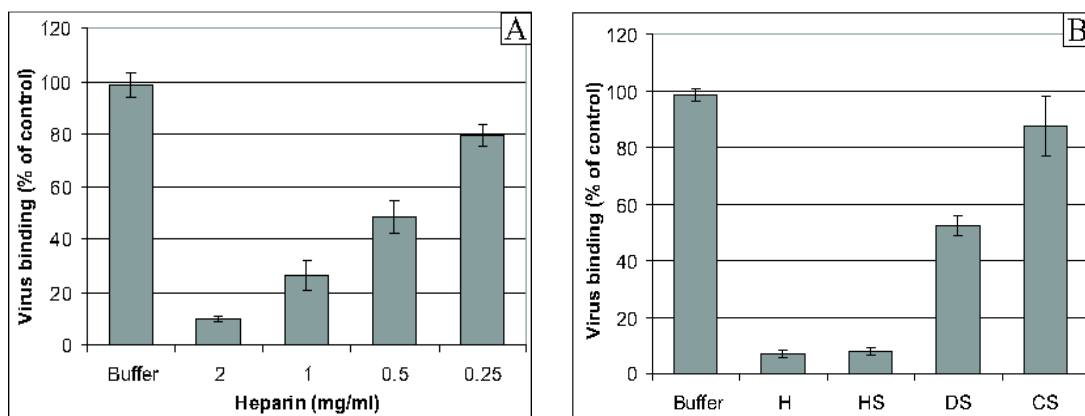
Figure 3.7: Heparin and HS inhibit HRV54 binding to RD cells. Cells were grown in 24 well plates, washed, and challenged with [^{35}S]-labeled virus at ~16,000 cpm in 0.4 ml HBSS in the presence of the GAGs with gentle rocking as indicated for 60 min at 34°C. Inhibition by heparin was assessed at concentrations between 0.25 and 2 mg/ml (A), and inhibition by the GAGs shown in (B) at 2 mg/ml; heparin (H); heparan sulfate (HS); dermatan sulfate (DS) and chondroitin sulfate (CS). Cells were washed to remove unbound virus and cell associated radioactivity was measured by liquid scintillation counting. Percentage virus binding in the presence of inhibitors to that of control (buffer) was calculated. Each value is an average of three independent experiments ± SD.

Sulfation is required for virus binding

Cell surface HS is a highly sulfated proteoglycan carrying negative charge. This character makes it very attractive candidate for interaction with bacteria and viruses possessing positive charge on the surface. From our infection inhibition assays, it became evident that highly sulfated heparin was a stronger inhibitor than HS. This finding clearly indicated that the interaction between HRV54 and HS is ionic in nature and sulfates on the HSPG play a major role. To support this idea, we carried out the suppression of sulfate incorporation into proteoglycans and tested its effects on virus binding. RD cells were grown for three days in medium with and without 50 mM NaClO$_3$. Cells were washed with HBSS and incubated with [^{35}S]-labeled virus at 16,000 cpm for 60 min. Unbound virus was removed and cell associated radioactivity was quantified by liquid scintillation counting. As shown in Fig. 3.8, HRV54 binding to cells grown in the presence of chlorate was reduced to 70% when compared to control cells. However, binding further diminished to about 90% in the presence of heparin.

This might be due to incomplete sulfate incorporation suppression by chlorates. Nevertheless, a strong decrease in virus binding to the chlorate treated cells clearly demonstrates the involvement of sulfated proteoglycan in virus interaction.
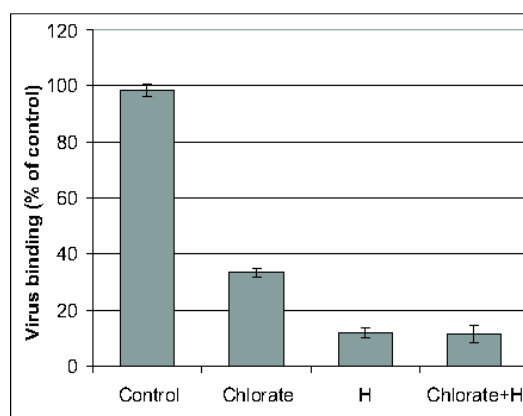

Figure 3.8: RD cells grown in medium containing 50 mM NaClO$_3$ exhibit reduced HRV54 binding. Cells were grown in medium with and without (control) 50 mM NaClO$_3$ for three days, washed, and incubated with virus at ~16,000 cpm for 60 min at 34°C. Unbound virus was washed away and cell associated radioactivity was quantified by scintillation counter. Binding is shown as % of the control. Each value is the average of three experiments ± SD.

Heparinase 1 treatment of RD-cells reduces HRV54 attachment

For further evidence that HRV54 interacts with cell surface HSPG, we used a more direct approach. Cell surface HS was enzymatically removed to observe effects on virus binding. RD-cells grown in 24 well plates were incubated with increasing concentrations of heparinase 1 for 2 h at 37°C, washed with ice cold buffer to remove digested material, and virus at 12,000 cpm was added. After 60 min incubation at 4°C, unbound virus was washed away and cell associated radioactivity was determined via scintillation counting. As seen in Fig. 3.9, virus binding was significantly decreased with increasing concentrations of the enzyme used to digest surface exposed HS. These results clearly demonstrate that HRV54 physically interacts with surface exposed HSPG.
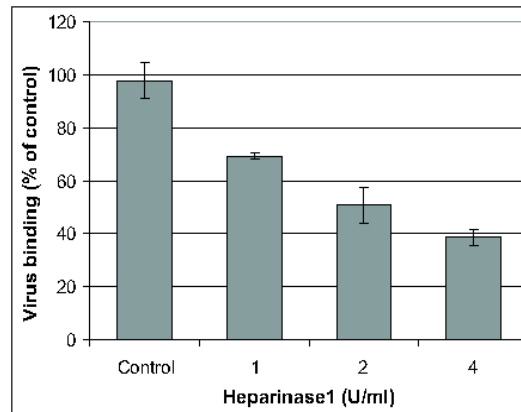
Figure 3.9: Heparinase 1 treatment of RD cells reveals a concentration dependent decrease in HRV54 binding. RD-cells were grown in 24 well plates, washed with HBSS, and incubated with the indicated concentrations of heparinase 1 for 2 h at 37°C. Digested material was removed with ice cold buffer, cells were challenged with radio-labeled virus at 16,000 cpm and incubation was extended for another h at 4°C. Unbound virus was washed away, cells were trypsinized, and cell associated radioactivity was determined. Control; virus bound to non-treated cells. Results are the mean from three independent experiments ± SD.

CHO-mutant cells deficient in proteoglycan synthesis fail to bind the virus

To further confirm the involvement of cell surface HSPG in virus binding, we made use of CHO mutant cells deficient in proteoglycan biosynthesis. These cells have been extensively used for the investigation of virus-HSPG interactions. CHO pgsA-745 cells are deficient in xylosyltransferase synthesis and are unable to produce any glucosaminoglycans, and pgsD-677 cells are doubly deficient in N-acetylglucosaminyltransferase and glucuronyltransferase and hence fail to synthesize HS but produce threefold higher levels of CS than the wild type. These cells along with CHO-K1 (wt) were seeded in 24 well plates, grown to 90% confluency, and washed with HBSS. [^{35}S]-labeled virus at ~12,000 cpm was added and the plates were incubated at 34°C for 60 min. Bound virus was determined as described above. A significant difference in binding to the different cell lines was evident (Fig. 3.10). Possessing both receptors, RD-ICAM cells strongly bound HRV54, followed by RD-cells devoid of ICAM-1. The virus significantly bound to CHO-K1 (wt) cells whereas the mutants showed

only a background binding. These results again confirm that HRV54 binds to cell surface HSPG.
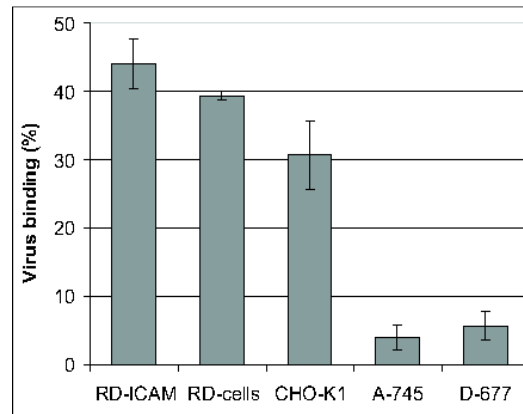

Figure 3.10: CHO mutant cells deficient in proteoglycan synthesis only show background binding of HRV54. Cells were seeded in 24 well plates, grown to 90% confluency, and washed with HBSS. [^{35}S]-labeled HRV54 at 12,000 cpm was added and the plates were incubated at 34°C for 60 min. Unbound virus was washed away. Cells were trypsinized and cell associated radioactivity and radioactivity in the supernatant was quantified by liquid scintillation counter. Percentage of bound virus to total input was calculated. Each value is a mean of three independent experiments ± SD.

HRV54 possesses double receptor specificity

ICAM-1 is the prime receptor for major group viruses. This receptor is not only responsible for cell attachment but also facilitates virus uncoating. We thus asked whether HRV54 can use both receptors independently. RD ICAM cells were grown in 96 well plates and incubated with R6.5 (an ICAM-1 specific monoclonal antibody that specifically blocks the virus binding site as exemplified with HRV14 (Norris et al., 1991)). On the other hand, HRV54 was incubated with heparin for 30 min at 34°C. Then, infection was monitored in the presence or absence of each, R6.5 and heparin alone, or in combination of both. After 24 h, cell survival was either viewed under microscope or quantified as in Fig. 3.5. As expected, neither R6.5 nor heparin alone prevented cell damage while in combination almost 100% of the cells

remained alive. These results clearly show that HRV54 can use either of the receptors for productive infection, depending on their availability.

To further investigate simultaneous involvement of both receptors in virus binding, RD-ICAM cells grown in 12 well plates, were preincubated with R6.5 and challenged with radiolabeled HRV54 in the presence or absence of heparin for 60 min at 34°C. Interestingly, no inhibition of virus binding was seen in the presence of R6.5, whereas heparin suppressed binding by 40%. This suggests that the remaining 60% binding is due to ICAM-1. However, when ICAM-1 is blocked then all viral particles bind to HS and there is no obvious inhibition. This is quite clear when both inhibitors were used completely abolishing virus binding all together.
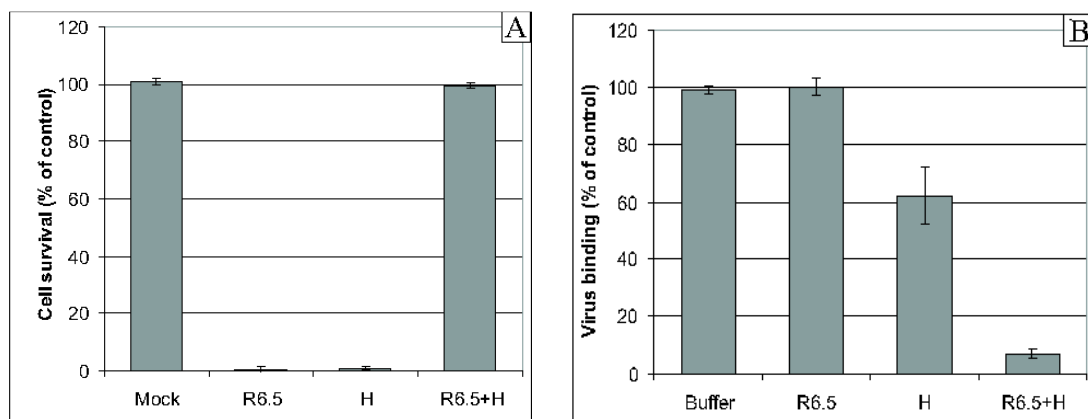
Figure 3.11: HRV54 can use ICAM-1 as well as HS independently. A) Only the combination of R6.5 and heparin completely protects RD-ICAM cells from viral damage. Cells grown in 96 well plates were incubated with R6.5 antibody to block ICAM-1 for 1 hour at 37°C. To block heparin binding sites, virus was incubated for 30 min at 34°C with heparin. Cells were infected with the virus in the presence or absence of R6.5 +/- heparin. After 24 h at 34°C, cell survival was determined as in Fig. 3.5. B) Only the combination of R6.5 and heparin completely blocks HRV54 binding to RD-ICAM cells. Cells grown in 12 well plates were incubated +/- R6.5 for 1 h at 37°C, washed and incubated with radio-labeled HRV54 +/-heparin for 60 min at 34°C. Virus binding as % of the control (C) is shown. Mean of three independent experiments ± SD.

HRV54 is inactivated below pH 5.2

A major characteristic of human rhinoviruses is their acid lability, which was used to differentiate them from enteroviruses (Hughes et al., 1974). HRVs usually become inactivated at pH < 3 (Stott and Killington, 1972). However, some rhinoviruses (e.g. HRV2) undergo structural changes associated with uncoating already at pH $\leq$5.6 and it is believed that all minor group viruses are uncoated at a pH around 5.5 which prevails in late endosomes. On the other hand, the catalytic activity of ICAM-1 allows infection of HRV14, HRV3, and HRV89 even in the presence of endosomotropic agents like bafilomycin A1 that increases the endosomal pH to neutrality (Bayer et al., 1999; Nurani et al., 2003; Vlasak et al., 2005a). Having observed the ability of HRV54 to infect RD cells lacking ICAM-1, we asked whether it is acid labile so that the low endosomal pH is sufficient to trigger the uncoating. Virus at 10^7 TCID$_{50}$ was incubated in sodium acetate buffer adjusted to pH 7, 6.5, 6.0, 5.6, 5.2, 4.8 and 4.4 for 30 min at room temperature. After re-neutralization with phosphate buffer the infectivity was determined. HRV2 and HRV89 were taken as controls representing each receptor group. As depicted in Fig. 3.12, HRV54 and HRV2 were inactivated below pH 5.6 while HRV89 remained infective even at pH 4.4 although at greatly reduced titer. Although this pH threshold for inactivation is below the pH encountered in late endosomes, the site of uncoating, it nevertheless indicates a similar sensitivity of HRV54 and HRV2. This suggests that usage of a receptor different from ICAM-1 requires the virus to be unstable at a pH prevailing in the endosomal system; this correlates well with previous data on the HRV89 mutants that, having acquired binding specificity for HS, also became less stable than wt (Vlasak et al., 2005a).
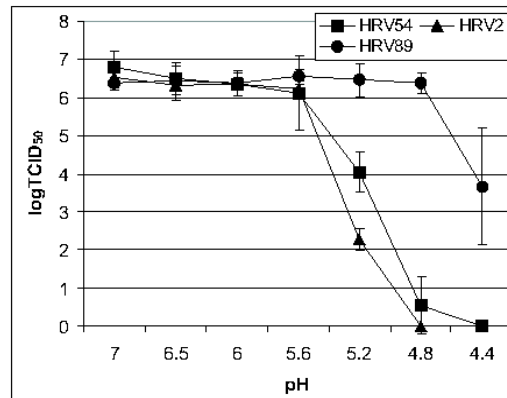
Figure 3.12: HRV54 is more sensitive to low pH than HRV89, another major group virus. HRVs were incubated at the pH indicated for 30 min at room temperature. The solutions were neutralized and virus infectivity was determined via end point dilution assay. HRV2 and HRV89 were taken as representatives for each group. Mean of three independent experiments ± SD.

HRV54 uncoating in the absence of ICAM-1 depends on low endosomal pH

To further investigate the role of the low endosomal pH in virus uncoating in the ICAM-1 negative cells, bafilomycin A1 (baf) was used. This drug inhibits the vesicular H^+-ATPase and has been extensively employed in investigations of the influence of the vesicular pH on viral infection (Bayer et al., 1999; Prchla et al., 1994; Vlasak et al., 2005a). For example, HRV2 infection is completely blocked in the presence of baf while infection with HRV14 proceeds albeit at lower efficiency (Bayer et al., 1999). This indicates that ICAM-1 catalyzed uncoating can occur at neutral pH. We thus conducted experiments to investigate the pH dependency of HRV54 infection. RD-cells were preincubated with baf for 60 min at 37°C, infected with HRV54, and incubation was continued for 24 h. As shown in Fig. 3.13A, almost 100% of the cells survived in the presence of the drug and the virus behaves very similar to HRV2 in this respect indicating the requirement of the low endosomal pH for uncoating. Carrying out the same experiments using RD-ICAM cells, it became clear that the strict requirement for the low pH is not met if ICAM-1 is present (Fig. 3.13B). However, when ICAM-1 was blocked with R6.5 these cells behaved similar to the cells without ICAM-1.
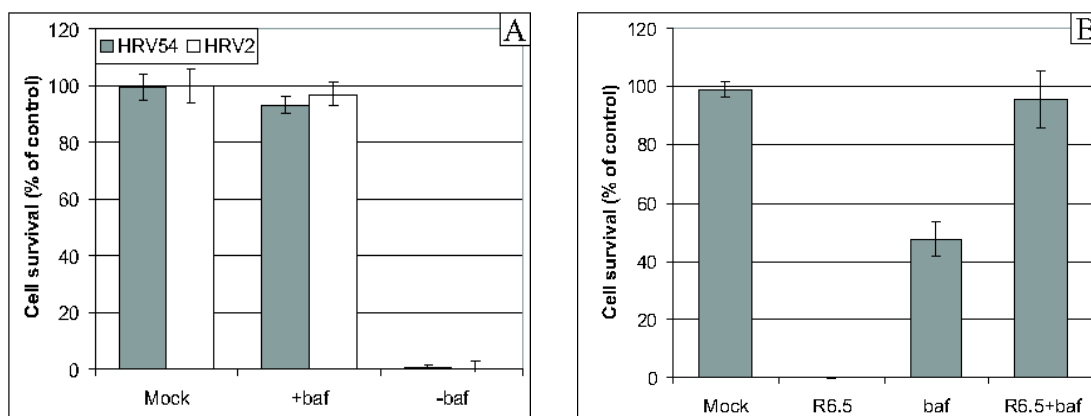
Figure 3.13: In the absence of ICAM-1 expression, HRV54 infection depends on the low endosomal pH. RD cells (A) and RD-ICAM cells (B) were preincubated with 200 nM of baf and infected with HRV54 and for control purposes with HRV2. R6.5 was also present during the experiment where indicated. Cell survival was monitored after 24 h as in Fig. 3.5. Mean values from three parallel experiments with +/- SD.

We further confirmed the dependency of virus uncoating on endosomal low pH by determining the viral titer in the presence or absence of the drug. Virus kinetics showed that the replication in the presence of baf took place in RD-ICAM cells (Fig. 3.14A) but not in RD cells (Fig. 3.14B). These results very well correlate with the previous data where presence of drug rescued the cells from viral damage.

Figure 3.14: Effect of baf on viral replication in RD-ICAM (A) and RD (B) cells. Cells were preincubated with or without baf and infected with HRV54 at 4°C for 1 h. Unbound virus was removed and

plates were shifted to 34°C. After the incubation times indicated cells were lyzed via freeze/thaw cycles and viral titer was determined from the supernatant. Mean values from three parallel experiments with +/- SD.
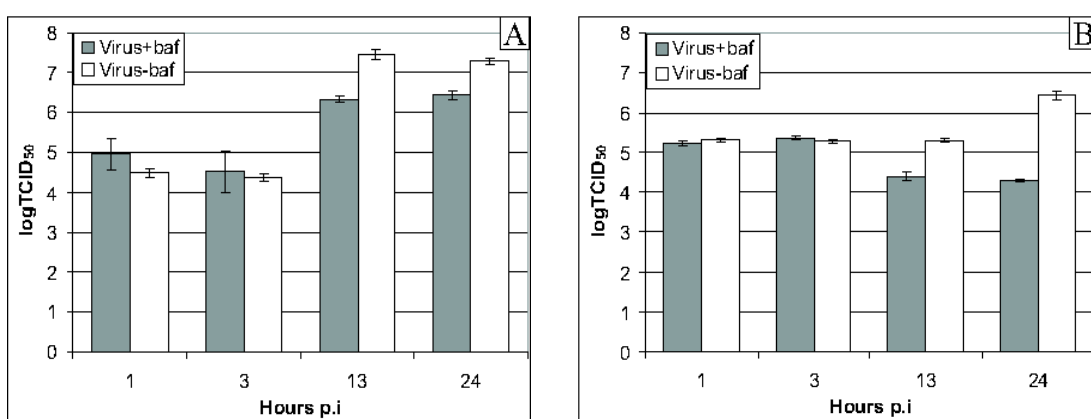
HRV54 entry and uncoating is less efficient than HRV2

Although HRV54 is able to infect the ICAM-1 negative RD cells via HS but the efficiency of infection is lower than in RD-ICAM cells. Due to this reason, we had to use 50 TCID$_{50}$/cell in all infection assays to see a prominent CPE. In order to rule out any cell type specific effects, we compared the efficiency of infection with HRV2. To this end we investigated the virus kinetics in RD cells. Virus was bound to the cells at 4°C and samples were frozen at the given time points. Upon virus titer determination after each respective time point, we observed a significant difference in the kinetics of both viruses. As shown in Fig. 3.15, HRV2 was readily uncoated upon entering the cells and lost almost 2 logs at 4 h p.i., whereas HRV54 showed significant slower uncoating. Due to this slow uncoating it lagged behind HRV2 through the whole time period. However, the total virus yield showed by HRV54 was essentially similar to HRV2 (8 logTCID$_{50}$) but only at 36 h p.i. (data not shown). These results clearly indicate that low efficiency of HRV54 infection is due to less efficient virus entry and uncoating. In RD-ICAM cells both viruses behaved essentially identical (data not shown).
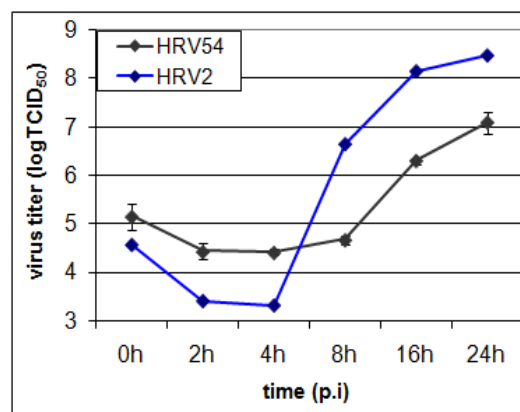

Figure 3.15: HRV54 exhibit slow kinetics in RD cells as compared to HRV2. RD cells were seeded in 24 well plates and infected with each virus at 10^6 TCID$_{50}$/well at 4°C for 1 h. Unbound virus was

removed by washing with ice cold PBS and the cells were over laid with infection medium and frozen at the given time point. Cells were lysed by three freeze/thaw cycles. Cell debris was removed by centrifugation and virus titer was determined via an end point dilution assay.

Wild type HRV8 binds heparan sulfate but does not infect

In order to further study the basis of the weak binding of HRV8 and HRV18 to V3X5 seen by VOB, we decided to use cell binding assays instead of western blot analysis. Interestingly, HRV8 showed significant binding to RD cells without causing infection and, the binding was not blocked by the presence of V3X5 but heparin and HS inhibited virus binding to RD cells. Carrying out essentially the same experiments previously described for characterization of HRV54 and the HRV89 variants, we found that HRV8 already exhibited HS-binding properties as the wild type. To test whether this binding also led to the entry, we used immunofluorescence microscopy to locate the virus in RD cells. Confocal microscopy identified the virions within the infected cells (Fig. 3.16A). However, the virus caused CPE and replicated in RD cells after only 3 blind passages alternating between RD and HeLa cells. Furthermore, it turned out that wild type virus was stable at the pH prevailing in the endosome as compared to these variants (HRV8v) selected upon passage (see above) and the efficiency of virus uncoating was apparently the major limiting factor in infection of RD cells. As seen from the *in vitro* inactivation experiment in (Fig. 3.16B), HRV8 wt lost less than one log infectivity at pH 5.8 whereas the titre of HRV8v was reduced by 4 logs at the same pH. Thus replication, but not uptake, in ICAM-1 negative cells correlates with decreased viral stability at low pH. This is taken to indicate that the more stable wild type, although entering the cell, cannot uncoat in the absence of the destabilizing activity of ICAM-1. The lower stability of HRV8v at acidic pH must therefore be the basis for productive infection. The mutation(s) resulting from the adaptation did not lead to loss of affinity for ICAM-1 as infection of HeLa or RD-ICAM cells was not blocked by heparin (data not shown). These results demonstrate that HRV8v must access acidic compartments for successful infection.
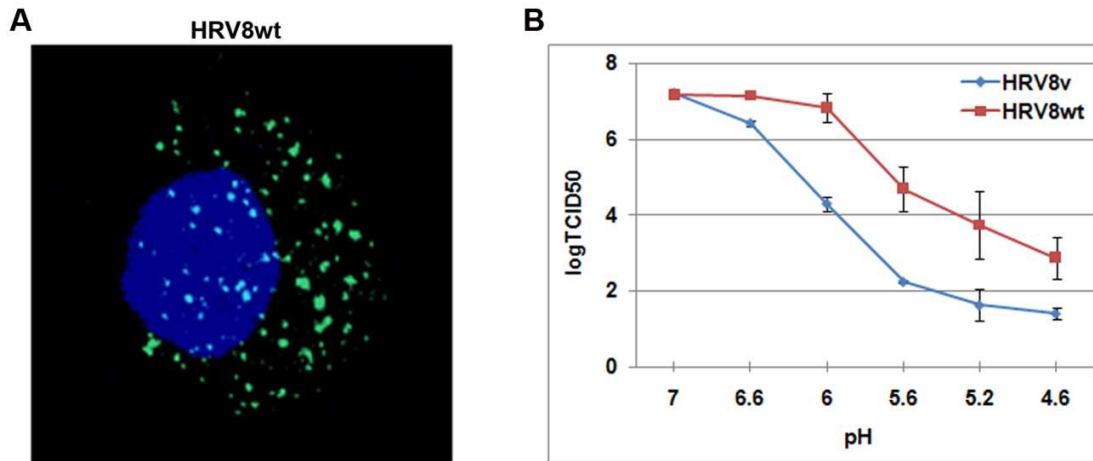
Figure 3.16: HRV8wt uncoating but not entry into RD cells correlates with its inability to infect ICAM-1 negative cells. A) RD cells were grown on glass cover slips and infected with HRV8wt at 300 $TCID_{50}$/cell at 34°C. After 30 min of continuous virus internalization, cells were washed and fixed with 4% PFA. HRV8 antiserum followed by alexafluor488 secondary antibody was used to trace the virus within the cells. Cells were scanned using LSM510 microscope. Nuclei were stained with DAPI. Ten individual stacks are combined. 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 infectivity was determined by endpoint dilution. Mean of 4 independent experiments ± SD.

Part 2 (Virus Entry Mechanisms via Different Receptors)

HRVs entering via different receptors accumulate at different locations within the cell

Our previous data suggested that not only virus binding but also entry and uncoating are crucial determinants for efficient and successful infections. After having found that HRVs can bind to three structurally and functionally entirely different receptors, it was of interest to follow the entry pathways taken by the viruses upon binding to each receptor. Therefore, we investigated the entry of each virus, binding to respective receptor by immunofluorescence microscopy. To this end, RD and RD-ICAM cells (transfected to stably express human ICAM-1 (Newcombe et al., 2003) were challenged with HRV2, HRV8v, and HRV14, respectively, for 30 min at 34°C. Immunofluorescence microscopy demonstrated entry of HRV2 and HRV8v into RD-cells, whereas, as expected, HRV14 did neither bind nor enter the cells lacking ICAM-1 (Fig. 3.17A, upper panels). HRV2 localized to small perinuclear vesicles; conversely, HRV8v was distributed all over the cell body in comparatively larger spots. In RD-ICAM cells, the pattern of HRV14 and HRV8v was essentially the same. However, the latter virus showed a substantial difference when using HS (in RD cells) as compared to when using ICAM-1 (in RD-ICAM cells) as receptor. In the former cells, the spots were larger and the strong signal seen at the plasma membrane of RD-ICAM cells was absent. The pattern of HRV2 was indistinguishable in the two cell lines. All three virus types also gave a very similar pattern in HeLa cells; nevertheless, the signal for HRV8v and HRV14 was also weaker when compared to the one seen in RD-ICAM cells overexpressing ICAM-1. Coinfection experiments (Fig. 3.17B) demonstrated that HRV2 did virtually not colocalize with HRV8v. However, there was some overlap of HRV2 with HRV14. On the other hand, HRV8v completely overlapped with HRV14 when internalized via ICAM-1 and with HRV89M, another major group virus adapted to use HS (Vlasak et al., 2005a), when internalized into RD cells. Again, in the latter case, the spots were clearly bigger. Taken together, this suggests that entry and intracellular trafficking of these HRVs is distinct and presumably only depends on the type of receptor used.
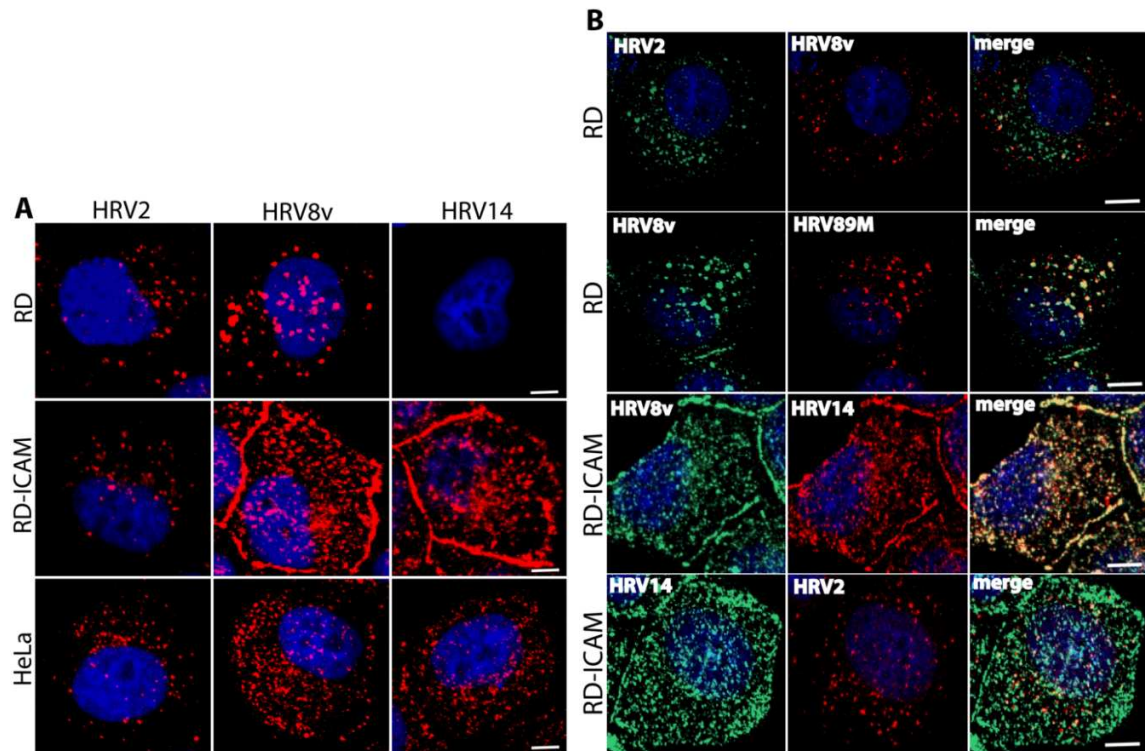
Figure 3.17: HRVs using different receptors exhibit different intracellular localization patterns. A) RD, RD-ICAM, and HeLa cells were challenged with the virus types as indicated at 300 $TCID_{50}$/cell for 30 min at 34^oC. Cells were washed, fixed, and the different virus types were stained with the respective type-specific antibodies, followed by TxR-labeled secondary antibodies and viewed under a confocal microscope. Nuclei were stained with DAPI. **B)** RD and RD-ICAM cells were coinfected with the viruses in combinations as given and were processed with essentially similar setup except appropriate labeled secondary antibodies were used to assess colocalization. Composite pictures of 10 confocal stacks are shown. Bar, 10 µm.

Virus entry via ICAM-1 and HS appears to be clathrin-independent

The different intracellular localization of HRV8v and HRV14 as compared to that of HRV2 made clathrin-dependent entry for these two virus types questionable. To confirm this interpretation, the respective viruses were co-internalized with human transferrin, a marker for clathrin-mediated endocytosis. RD-ICAM cells were incubated with HRV14 at 4^oC in the presence of Alexa Fluor 568-transferrin for 1 h, cells were washed, shifted to 37^oC, and fixed at 0, 5, and 15 min of incubation. Confocal microscopy revealed that most of the transferrin was internalized and located either in early endosomes or in the perinuclear region at 5 min

after temperature shift while HRV14 largely remained at the cell periphery (Fig. 3.18A). At 15 min, transferrin had accumulated in the perinuclear region whereas HRV14 was evenly distributed throughout the cell body with a substantial fraction still remaining on or close to the cell surface. Notably, there was no co-localization at any time and HRV14 was in different patches even at the plasma membrane (at 0 min of internalization). These data clearly demonstrate that transferrin and HRV14 enter with different kinetics and localize to different compartments, making clathrin-mediated endocytosis for HRV14 highly unlikely.

Entry of HRV8v via HS was studied under similar conditions but using RD cells. However, because of weak virus binding at low temperature, we had to employ continuous internalization for the times indicated in Fig. 3.18B instead of pulse-chase conditions. HRV8v behaved similarly to HRV14 with respect to lack of co-localization with transferrin, entry kinetics, and intracellular trafficking (compare to Fig. 3.18A). This result also rules out clathrin-dependent entry for HRV8v via HS. HRV2, used as a control, completely overlapped with transferrin (Snyers et al., 2003) and Fig. 3.18C), although their intracellular pathways separate rapidly (Brabec et al., 2006).

More direct proof for clathrin-independent entry via ICAM-1 and HS was obtained using a GFP-tagged clathrin light chain. RD-ICAM and RD cells overexpressing GFP-clathrin were challenged with virus and fixed at 5 and 15 min p.i. In accordance with the previous results, GFP-clathrin did not at any time colocalize with HRV8v or with HRV14 (Fig. 3.18D and E) but again HRV2 and transferrin used as controls showed substantial colocalization or complete overlap respectively with GFP-clathrin (Fig. 3.18F and G). These results clearly indicate the mode of entry via LDLR as clathrin-dependent while via ICAM-1 and HS as clathrin-independent.

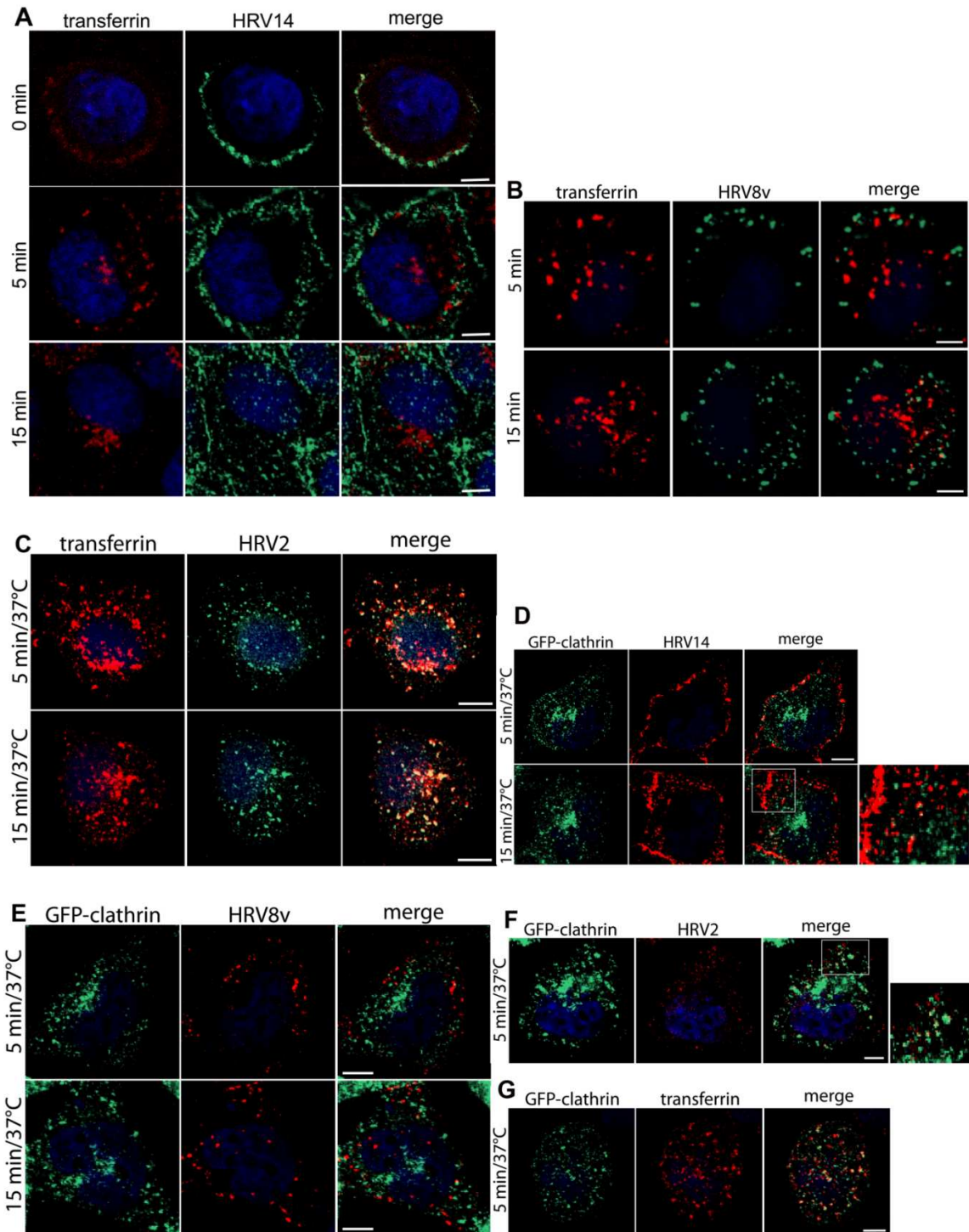**Figure 3.18: HRV14 and HRV8 do not colocalize with transferrin or GFP-clathrin. A)** RD-ICAM cells were incubated with HRV14 at 300 TCID$_{50}$/cell and 10 µg/ml Alexa Fluor 568-transferrin for 60 min at 4°C. Unbound material was washed away and cells were shifted to 37°C. At the times indicated, cells were fixed and stained for virus with type-specific mouse antiserum followed by Alexa Fluor 488-labeled

secondary antibody. Colocalization was assessed by confocal fluorescence microscopy. The lower panels are composite pictures of 10 confocal stacks. **B&C**) RD cells were incubated with HRV8v and HRV2 at 500 TCID$_{50}$ /cell and 10 µg/ml Alexa 568-transferrin for 5 and 15 min at 37°C (continuous internalization) and were processed and viewed as in Fig. 3.17. **D-G**) RD-ICAM and RD cells were transfected with GFP-clathrin plasmid. After 48 h of transfection, cells were infected with HRV14 (D) and HRV8v respectively for the time indicated (E). Each virus was detected type specifc antibodies followed by TxR secondary antibody. Cells were processed and viewed as above. HRV2 (F) and transferrin (G) were used as controls. Bar, 10 µm.

Dominant negative inhibitors of clathrin-mediated endocytosis do not affect virus entry via ICAM-1 and HS

For further confirmation of the lack of clathrin-dependence, we used dominant-negative (DN) mutants that potently interfere with the normal functions of key proteins of the clathrin-dependent pathway. The SH3 domain of amphiphysin (amphi-SH3) has been shown to inhibit receptor mediated endocytosis of HRV2, transferrin, and epidermal growth factor (Snyers et al., 2003; Wigge et al., 1997) amongst many other ligands by interfering with recruitment of dynamin to coated pits. Accordingly, RD-ICAM cells were transfected with myc-tagged amphi-SH3 followed by infection with HRV14. Confocal microscopy revealed no difference in entry regardless of the expression of amphi-SH3 (Fig. 3.19). Amphi-SH3 was shown to prevent self assembly of dynamin rings, which impedes constriction of clathrin-coated pits but does not affect clathrin assembly by itself (Owen et al., 1998). Thus, to rather more directly assess clathrin function, we also used the C-terminal domain of AP180 (AP180-C), which affects clathrin-mediated endocytosis without interfering with dynamin functions (Ford et al., 2001). Again, overexpression of DN AP180-C failed to modify entry of HRV14; In contrast, transferrin uptake was prevented by both DN inhibitors. The same experiments were also carried out with HRV8v in RD cells with essentially the same results (data not shown).
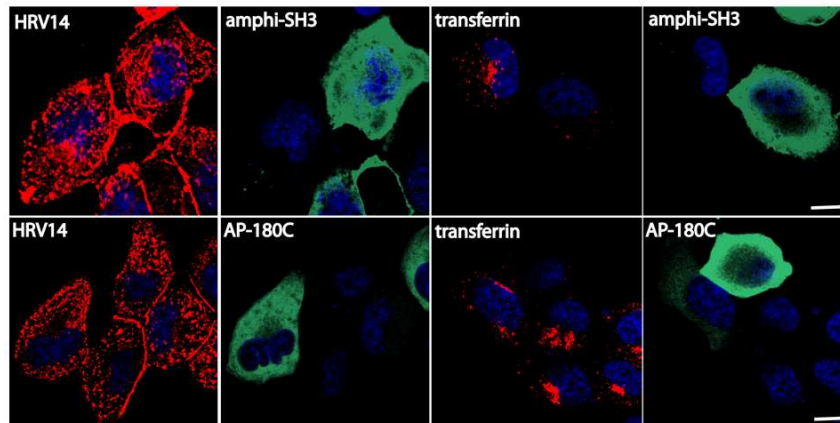
Figure 3.19: Dominant negative inhibitors of the clathrin-dependent pathway do not affect HRV14 entry. RD-ICAM cells were transfected with DN myc-tagged amphi-SH3 and AP180-C as indicated and challenged with HRV14 at 300 $TCID_{50}$/cell for 30 min at 37^oC (continuous internalization). Virus was detected with type-specific antiserum and TxR-labeled secondary antibody, whereas expression of the DN inhibitors was assessed by using mouse anti-myc mAb followed by Alexa Fluor 488-labeled secondary antibody; samples were processed and viewed as in Fig. 3.17. One confocal slice is shown. Bar 10 µm.

HRV14 and HRV8v do not colocalize with cholera toxin B

The caveolin pathway is the second most well characterized cell entry route. Involvement of caveolin was evaluated by assessing a possible co-localization of virus with cholera toxin subunit B (CtxB). CtxB is a widely accepted marker of this pathway although it has also been shown in association with flotillin- (Glebov et al., 2006) and clathrin-dependent endocytosis in various cell types (Hansen et al., 2005; Torgersen et al., 2001). RD-ICAM cells were incubated with HRV14 in the presence of Alexa Fluor 488 CtxB for 60 min at 4^oC, washed, and shifted to 37^oC for the times indicated in Fig. 3.20A. Confocal microscopy showed some overlap of HRV14 with CtxB at the cell surface at 0 min and little bit more after shifting to 37^oC for 5 min, but on further internalization they separated from each other. CtxB moved to the Golgi and concentrated at the perinuclear region at 15 min after the temperature shift while the HRV14 signal remained spread throughout the cytosol. These results indicate that HRV14 and CtxB enter with different kinetics and use different pathways to different destinations within the cell.

To investigate entry via HS, RD cells were infected with HRV8v in the presence of CtxB as above and fixed at 5 and 15 min of continuous internalization at 37°C. Confocal images again showed very little colocalization of virus and CtxB (Fig. 3.20B). It is noteworthy that even after 1 h virtually no colocalization was apparent, thus excluding transport to caveosomes; SV40, a recognized marker of caveosomes arrives in these organelles after 40 min (Pietiainen et al., 2004). Further confirmation by more direct means was obtained via staining caveolin-1 and virus with the respective antibodies. Neither HRV8v nor HRV14 colocalized with caveolin-1 (Fig. 3.20C). These results exclude the involvement of caveolin-1 in viral entry and intracellular trafficking via HS and ICAM-1.
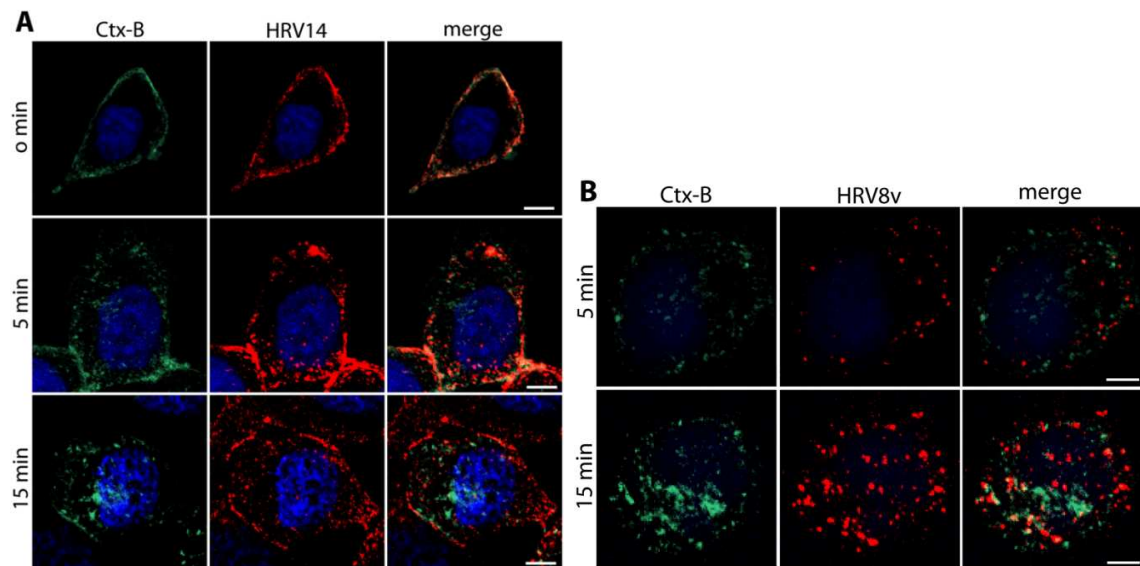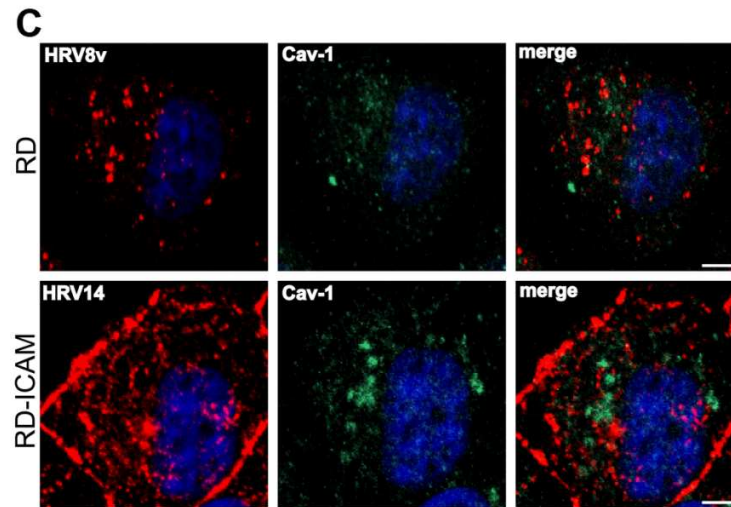
Figure 3.20: HRV14 and HRV8v neither colocalize with cholera toxin B nor caveolin-1.
A) RD-ICAM cells were incubated with HRV14 at 300 $TCID_{50}$ /cell and 1 µg/ml Alexafluor488-CtxB for 60 min at 4^oC. Unbound material was washed away and cells were shifted to 37^oC. At the times indicated cells were fixed and stained for virus with type-specific mouse antiserum followed by TxR-labeled secondary antibody. Colocalization was assessed by confocal fluorescence microscopy. **B**) RD cells were incubated with HRV8v at 500 $TCID_{50}$ /cell and 1 µg/ml Alexafluor488-CtxB for 5 and 15 min (continuous internalization) and were processed as above. **C**) RD and RD-ICAM-1 cells were infected with HRV8v and HRV14 respectively and were fixed after 30 min of continuous virus internalization. Caveolin-1 was detected using rabbit anti-caveolin-1 antibody followed by alexafluor488-lebeled secondary antibody. Detection of virus was carried out essentially same as above. Colocalization was analyzed using confocal microscopy. Bar 10 µm.

Entry via ICAM-1 and HS is flotillin-1 independent

Flotilin-1 and -2 have been described as markers of a clathrin and caveolin-independent pathway (Glebov et al., 2006) and more recent data suggested involvement of flotillin-1 in uptake of HS-binding ligands (Payne et al., 2007). Having excluded entry of HRV14 and HRV8v via clathrin and caveolin-dependent routes, it was of interest to investigate whether flotillin-1 was involved. Again, by using fluorescence microscopy, colocalization with flotillin-1 was neither seen for HRV14 entry via ICAM-1 (Fig. 3.21A) nor for HRV8v internalized via HS (Fig. 3.21B). Therefore, it is very unlikely that these viruses follow a flotillin-1 dependent route when accessing the cell via ICAM-1 as well as via HS.
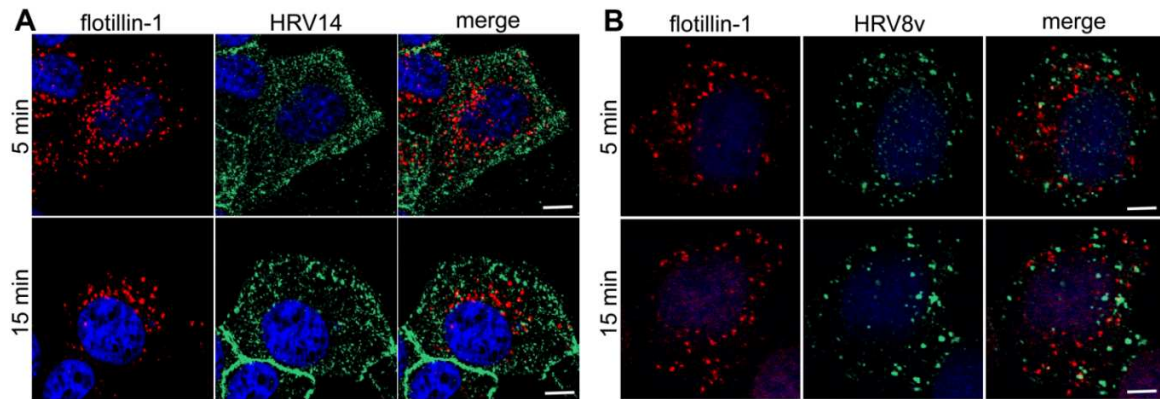
Figure 3.21: Flotillin-1 does not colocalize with HRV14 and HRV8v. RD-ICAM and RD cells were infected with HRV14 and HRV8v, respectively at 300 $TCID_{50}$ /cell for 5 and 15 min at 37^oC, unbound virus was removed, the cells were fixed. Flotillin-1 was stained with rabbit anti-flotillin-1 antibody followed by TxR-conjugated secondary antibody. HRVs were detected with type-specific antibodies followed by Alexafluor488-labeled secondary antibody. Cells were processed and viewed as in Fig. 3.17. Bar 10 µm.

Viruses entering via ICAM-1 and HS induce uptake and colocalize with FITC-dextran

Finally, we studied macropinocytosis as a candidate pathway for viral entry. RD and RD-ICAM cells were challenged with HRV2, HRV8v, and HRV14, respectively, in the presence of 500 µg/ml of the fluid-phase marker FITC-dextran for 15 and 30 min at 34^oC. Cells were fixed and examined by confocal fluorescence microscopy. Fig. 3.22 shows substantial colocalization of HRV14 (A) and HRV8v (B) with FITC-dextran, a marker recognized to be relatively specific for macropinocytosis (Falcone et al., 2006). HRV2 did not colocalize with FITC-dextran to any significant extent but three virus types strongly induced the uptake of the marker (not shown). These results suggested that uptake via ICAM-1 as well as via HS occur via macropinocytosis.
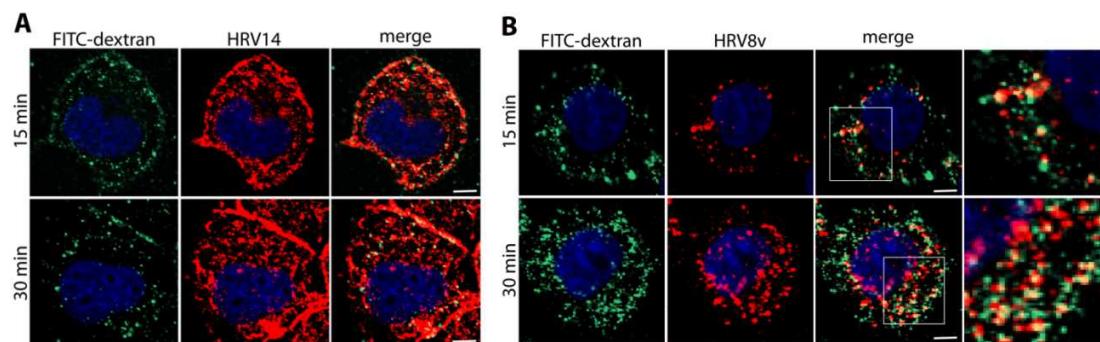
Figure 3.22: FITC-dextran colocalizes with HRV14 and HRV8v. RD-ICAM (A) and RD cells (B) were incubated with virus at 300 $TCID_{50}$ /cell in the presence of 500 µg/ml of FITC-dextran for 15 and 30 min at 34°C, washed, and fixed and processed for immunofluorescence microscopy as in Fig. 3.17. Bar 10 µm.

Pharmacologic inhibitors differently affect entry of HRV2, HRV8v, and HRV14

Finally, we studied the effect of various drugs that have been extensively employed to specifically inhibit endocytosis pathways. In order to avoid exposure of the cells to the chemicals for extended times, as is required to detect virus production, we utilized cleavage of the eukaryotic translation initiation factor eIF4GI as readout. Upon uncoating, the viral RNA is released into the cytoplasm and is directly translated into a polyprotein; autocatalytic cleavage sets free the viral proteinase 2A that efficiently cleaves eIF4GI into fragments detectable even at 3 h post infection. RD and RD-ICAM cells were pre-incubated with the respective drugs for 30 min, challenged with HRV2, HRV8v, and HRV14, as indicated in Fig. 3.23, and collected after 3 h. As previously described for poliovirus (Irurzun and Carrasco, 2001), processing of eIF4GI was assessed via Western blot analysis.

Chlorpromazine has been extensively used to demonstrate clathrin-mediated endocytosis of viruses (see for example (Akula et al., 2003; Raghu et al., 2009). Although this compound is known to possess a number of side effects, it is still a good choice when entry of different ligands into the same cell type is studied. In agreement with the results above, eIF4GI cleavage upon HRV2 infection was prevented by the drug, but was virtually unaffected upon infection with HRV8v and HRV14.

Caveolin-mediated endocytosis is associated with lipid rafts. Removal or sequestering of cholesterol profoundly inhibits entry of CtxB and other ligands internalized via this route (Schnitzer et al., 1994). Filipin destroys the rafts by intercalating between cholesterol molecules while methyl-β-cyclodextran (MβCD), one of the widely used inhibitors, is a heptasaccharide possessing hydrophobic core that interacts with cholesterol and hence drug easily extracts the cholesterol from the membrane (Irie et al., 1992). Both result in impairment of caveolin/lipid raft-dependent endocytosis. Filipin showed no effect on HRV14 and HRV2 but was slightly inhibitory on HRV8v. Similarly, MßCD only marginally affected HRV14 and was without noticeable effect on HRV2. However, it strongly decreased eIF4GI cleavage caused by HRV8v infection. This confirms the absence of involvement of caveolin/lipid rafts in endocytosis of HRV2 and HRV14 but suggests that lipid rafts plays a minor role in HRV8v entry. However, it must be noted that MβCD can inhibit CME and raft-dependent macropinocytosis, the effects of filipin are much more specific on caveolar endocytosis (Monis et al., 2006; Smart and Anderson, 2002). Thus, the inhibition of HRV8v by MβCD is most probably due to its involvement in macropinocytosis.

Amiloride, an inhibitor of the Na^+/H^+ exchanger, and cytochalasin D, which disrupts actin filaments, have both been shown to inhibit macropinocytosis. Although the mechanism of action of amiloride on macropinocytosis is still unclear, it has been employed to study entry of various viruses and other ligands at mM concentrations (Karjalainen et al., 2008; Schneider et al., 2007; West et al., 1989); cytochalasin, on the other hand inhibits membrane ruffling and re-organization required for plasma membrane engulfment of liquid and ligands. Amiloride inhibited cleavage of eIF4GI by all three virus types. While these results agree well with a macropinocytic pathway of HRV8v and HRV14, they disagree with the clathrin-dependence of HRV2 internalization. However, immunofluorescence microscopy unequivocally demonstrated that the drug did not prevent cell entry of HRV2 even at 5 mM but cleavage was completely inhibited at 200 µM, a concentration that failed to block HRV14 and HRV8v entry and cleavage of eIF4G; this points to steps different from endocytosis (such as RNA release and/or translation) being targeted by amiloride. Cytochalasin affected HRV2 strongly and HRV8v somewhat less; there was only marginal influence on HRV14. Fluorescence microscopy demonstrated that HRV8v and HRV14 remained on the plasma membrane in the presence of amiloride or cytochalasin D regardless of the receptor used indicating a direct

effect of the drugs on viral uptake. In contrast to amiloride, cytochalasin inhibited HRV2-entry as well (not shown). This effect might be explained by the well known involvement of actin in clathrin-mediated entry (Yarar et al., 2005).

Finally, dynasore, an inhibitor of the GTPase activity of dynamin (Macia et al., 2006), prevented eIF4GI cleavage upon infection of the cells with HRV2 and HRV8v but not with HRV14. Where this result is well consistent for clathrin-dependent entry of HRV2, it also points to dynamin being involved in macropinocytic entry of HRV8v. However, it disagrees with earlier findings of dynamin-dependent entry of HRV14 into HeLa cells (DeTulleo and Kirchhausen, 1998) and indicates that requirement of dynamin in macropinocytosis of ICAM-1 bound ligands is cell-type specific.
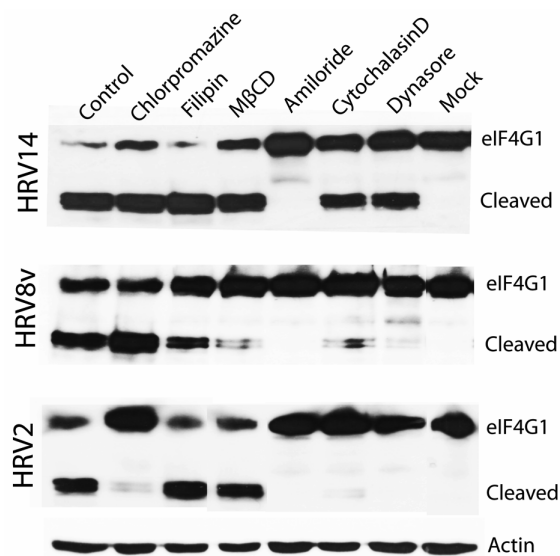
Figure 3.23: Inhibition profile of pharmacological inhibitors. Cells were grown in 24 well plates until >90% confluent. The drugs were added at the concentrations indicated in Materials and Methods and further incubated for 30 min. Then, virus was added at 300 $TCID_{50}$ /cell and incubation continued for 3 h. After removal of the supernatant, the cells were harvested in sample buffer and proteins separated on a SDS 6% polyacrylamide gel. Cleavage of eIF4GI was monitored via western blotting with a rabbit antiserum followed by HRP-conjugated anti-rabbit antibodies and proteins were detected by chemiluminescence recorded on X-ray film. Note that the extent of cleavage under control conditions (i.e. absence of inhibitors) differed for the three virus types; actin was used as a loading control.

Electron microscopy of virus entry

The tubovesicular compartments involved in the diverse entry pathways exhibit characteristic morphological features and clathrin-coated pits, caveolae, and macropinocytic membrane invaginations are distinguishable by EM. In addition, structures morphologically different from the three mentioned above have been recently implicated in other uptake mechanisms (Lundmark et al., 2008). We thus studied entry of the three HRV types by electron microscopy. RD cells were infected with HRV2 and HRV8v, and RD-ICAM cells with HRV14 for the times indicated in Fig. 3.24 and thin sections were prepared. As expected, HRV2 accumulated in coated pits and vesicles at 5 min pi. Conversely, many HRV14 virions appeared aligned in tubular structures like pearls on a string. This is very similar to the structures observed previously in HRV14-infected BHK cells transfected to express human ICAM-1 (Grunert et al., 1997) and in adenovirus 3 - infected HeLa cells (Amstutz et al., 2008). HRV8v was neither seen in coated pits/vesicles nor in the tubular structures but was rather found in shallow invaginations of the plasma membrane. Although apparently exploiting macropinocytosis, HRV8v and HRV14 thus enter via morphologically different membrane invaginations.

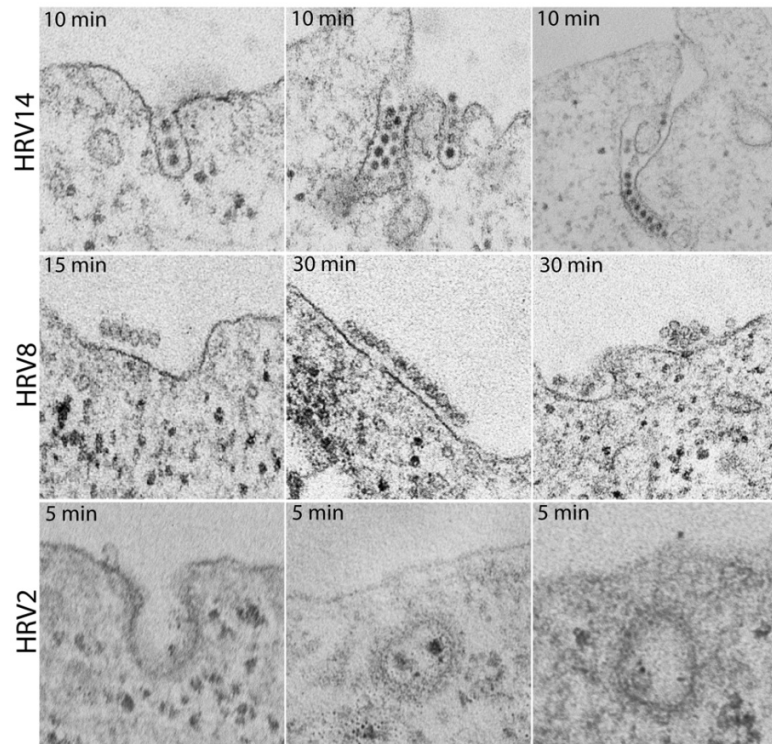**Figure 3.24: Negative stained thin section electron microscopy of virus entry.** RD-ICAM and RD cells grown on cover slips were infected with HRV14, and HRV8 at 10,000 $TCID_{50}$ /cell for the times indicated at 34^oC. Cells were fixed and stained with OsO_4, embedded in resin, and cut into 70 nm slices. They were viewed at 28,000 x magnification. Entry of HRV2 into HeLa cells is shown as a control.

4. Discussion

PART 1

HSPG as an Alternative Receptor

Although phylogenetically very similar and causing the same disease, the many rhinovirus types use two structurally and functionally unrelated receptors for infection and follow distinct pathways for uncoating (Bayer et al., 1998; Schober et al., 1998). In particular, a single lysine residue in the HI loop of VP1 of HRV2 is essential in the interaction with members of the LDL-receptor family (Verdaguer et al., 2004). These receptors bind close to the five-fold symmetry axis whereas ICAM-1 binds within the viral canyon of major group HRVs (Olson et al., 1993). The lysine residue is strictly conserved in all 12 minor group HRVs and is also present in 9 serotypes of the major group of rhinoviruses (Vlasak et al., 2003); yet, neither of these latter was able to use LDL-receptors for infection. This indicates that the lysine is necessary but not sufficient for receptor recognition (Vlasak et al., 2005b). Re-examination of the receptor specificity of these K-type HRVs via western blot analysis revealed that HRV8 and HRV18 significantly bound to MBP-V33333 (V3X5) a recombinant concatemer of repeat 3 of VLDLR (that strongly neutralizes all minor group HRVs) or MBP-V1-8 (complete VLDLR) although this interaction was much lower than when compared to HRV2. The low affinity was further confirmed when LDLR protein was used instead of the concatemers. Thus binding of the K-type HRVs to V3X5 could result from the high local concentration of the protein on the membrane as no interaction was observed in solution. Interestingly, bioinformatics energy calculations virus-receptor models indeed identified these viruses to fall close to the threshold values for minor group HRV2. Thus some of the K-type HRVs interact with minor group receptors but this interaction is too weak to result in infection of ICAM-1 negative cells. Moreover, major group HRVs not only require ICAM-1 for binding and entry, but also for uncoating. It could very be that even if a virus can bind and enter, due to lack of uncoating it is not able to infect the cells.

Apart from western blot analysis, we also screened the K-type HRVs for their ability to infect ICAM-1 negative RD cells. To our surprise it turned out that HRV54 was able to infect these

cells although the efficiency of infection was low. Since infection by this serotype was not inhibited by MBP-V33333 and no receptor binding was evident in VOB, involvement of LDL-receptors in cell attachment was excluded. We thus investigated cell surface heparan sulfate as a likely candidate receptor. Indeed, heparin as well as HS strongly inhibited HRV54 induced CPE in RD cells in a concentration dependent manner and complete protection was seen at concentrations exceeding 1 mg/ml. This inhibitory concentration is comparable to that determined for HS binding echoviruses which require between 125 µg/ml and 2 mg/ml of heparin to completely abolish infection (Goodfellow et al., 2001). However, requirement of comparatively high concentration might also reflect the fact that we had to use a higher input virus titer (50 $TCID_{50}$/cell) to observe CPE due to low efficiency.

The interaction between HRV54 and the glycosaminoglycan is relatively specific since dermatan sulfate and chondroitin sulfate were only marginally effective in preventing virus binding. Furthermore, sulfate modification of the glycan is required since growth of the cells in the presence of chlorate substantially reduced attachment of HRV54. The involvement of HS in virus binding was further supported by treatment of the cells with heparinase 1 and the use of CHO mutants with defects in glycan synthesis; virus attachment was drastically reduced upon enzymatic removal of heparan and CHO A-745 and D-677 cells that are defective in heparan sulfate synthesis, only showed background binding.

Re-examining HeLa-H1 cells that are commonly used for propagation of HRVs, we found that the cells were indeed killed by HRV54 in the presence of R6.5, a monoclonal antibody that efficiently blocks ICAM-1 (Reischl et al., 2001). This antibody has been previously used to assess receptor specificity of the K-type viruses but in these experiments infection was carried out at $TCID_{50}$ of 10^3 per well (Vlasak et al., 2005b); in the present experiments a $TCID_{50}$ of 10^5 was used, demonstrating that the efficiency of infection via HS is low and detection requires challenging at much higher virus concentrations than is the case for cells expressing ICAM-1. Preincubation of HRV54 with heparin (to block its heparin binding sites) together with blocking ICAM-1 on the cell surface with R6.5 led to complete inhibition of infection indicating that receptor usage is limited to ICAM-1 and heparan sulfate. This makes the involvement of an additional receptor, including LDL-receptors, unlikely. Interestingly, R6.5 did not appreciably diminish binding of radiolabeled virus to RD-ICAM cells, whereas

heparin reduced virus attachment by about 40%. Apparently, the ICAM-1 molecules are limiting in virus binding whereas HS molecules are not.

ICAM-1 facilitates uncoating, presumably by stabilizing an intermediate conformation state of the capsid. Furthermore, RNA release also occurs in the presence of lysosomotropic agents and drugs that neutralize the endosomal pH (Nurani et al., 2003). Since LDL-receptors lack such an activity, the minor group virus HRV2, and most probably all members of the minor group, are strictly dependent on the low pH environment for uncoating. We thus asked how HVR54 was uncoated in the absence of ICAM-1 and determined its pH stability. Comparison with HRV2 and the major group HRV89 revealed that it exhibits a similarly low acid stability as HRV2. The threshold pH for HRV2 inactivation was somewhat lower than the one previously determined (Gruenberger et al., 1991), which might be due to different experimental setups, nevertheless, it is evident that HRV2 and HRV54 exhibit similar acid sensitivity profiles. Thus, like HRV2, HRV54 should be completely blocked by the vesicular H^+-ATPase inhibitor (bafilomycin A1) in the absence of ICAM-1. This was indeed the case.

A number of enteroviruses have been shown to use HS as an alternative receptor for cell entry; since many of them normally attach to members of the immunoglobulin superfamily that bind within the viral canyon and aid in uncoating, it is not clear how the RNA of these acid-stable viruses becomes released within the cell. Are there additional factors catalyzing uncoating? It would be interesting to examine the heparan sulfate-specific enteroviruses for their pH stability. They might be more labile than those that exclusively bind ICAM-1, the poliovirus receptor, or the coxsackie adenovirus receptor.

Finally, we compared the efficiency of infection via the two different receptors. The virus yield was consistently by about 1 order of magnitude lower when ICAM-1 negative cells were infected and appreciable viral *de novo* synthesis was clearly seen only at 24 h p.i. whereas it was virtually finished within 13 h in RD-ICAM cells. This went hand in hand with cell death; no RD-ICAM cells were left at 13 h but some few intact RD cells were still found at 24 h. This fact can be tentatively explained by assuming that the uncoating is less efficient or occurs at an unfavorable site within the endocytic pathway when ICAM-1 is absent. This became more evident when we compared HRV2 and HRV54 virus binding, entry, uncoating, and yield in RD cells. Indeed, we found that the lower efficiency of HRV54 actually stemmed

from its slow entry and inefficient uncoating in these cells. However virus production by these cells was virtually identical but only in case of HRV54 it took longer (36 h) than HRV2 (Fig. 3.15).

In summary, our results demonstrate that HRV54, a major group human rhinovirus, is able to use heparan sulfate in addition to ICAM-1 for infection. In contrast to HRV89 that required extensive adaptation to acquire HS-binding (Reischl et al., 2001; Vlasak et al., 2005a), this property is intrinsic to wt HRV54. Although there is abundant HS on the cell surface and attachment appears to be difficult to saturate, infection via the proteoglycan is much less efficient than via ICAM-1 that is present at much lower concentrations. This points to differences in virus uptake, uncoating, and/or in routing to the site most efficient for RNA release and might explain why the ubiquitously and extensively expressed heparan sulfate is not a good receptor for HRV54 for efficient infection.

Selection of HRV8 Variants and their Characterization

From cell binding assays we found that HRV8 could also bind equally well to RD and RD-ICAM cells. However, this type was not able to cause any CPE to RD cells. Using similar experiments as carried out with HRV54, it turned out that this type also uses HS for binding to RD cells. Interestingly the virus was able to enter the cells as revealed by immunofluorescence microscopy but could not infect due to lack of uncoating. However, when infecting with higher MOI and leaving cells for longer, we could see some plaques resulted from variants. Thus after passaging this virus alternatingly between RD to Hela, we obtained variants which could efficiently infect and replicate in RD cells. These variants, as expected, are acid labile and readily inactivated at pH 5.6 while wt virus was considerably stable at this pH. This strongly support the idea that virus uncoting but not entry is responsible for lack of infection of RD cells by HRV8wt. Thus, our results provide convincing evidence that apart from receptor selection and entry, virus uncoating is also a major determinant of successful infection. Furthermore, HS-binding viruses apart from mutations causing a switch in receptor also need to alter their capsid stability for successful infection in the absence of catalytic receptor ICAM-1. Perhaps this might be a reason for HS-binding mutants not being

as common in rhinoviruses as in FMDV and other virus families where low endosomal pH alone is sufficient to destabilize the virus particles.

Part 2

Macropinocytosis and Clathrin-dependent Uptake of Human Rhinoviruses: Usage of Different Receptors Leads to Different Entry Pathways

Cell-penetrating peptides, such as polyarginine, antennapedia, the HIV TAT protein, and many other ligands have been shown to bind to and be taken up via HS (Fuchs and Raines, 2004; Ram et al., 2008). To this list of ligands, an ever increasing number of microbes and viruses is being added that are exploiting this receptor for cell attachment and entry (Chen et al., 2008; Fuchs and Raines, 2004; Ram et al., 2008). However, data on the uptake pathway of these and other ligands are controversial. Evidence has been presented for their delivery into the cell being dependent on clathrin, caveolin or occurring via macropinocytosis. Other reports even exclude endocytic uptake altogether (for a summary see (Poon and Gariepy, 2007)). In addition, a new, flotillin-dependent entry pathway was described for some of the HS-ligands. However, it is not yet clear whether this just constitutes a special case of non-clathrin non-caveolin endocytosis or has to be considered a new endocytosis route altogether (Payne et al., 2007). With respect to picornavirus entry it is notable that a HS-binding foot-and mouth disease virus (FMDV) variant was demonstrated to exploit a caveolin-dependent entry pathway (O'Donnell et al., 2008).

Having identified HS as an alternative receptor for some major group HRVs, such as HRV54 (Khan et al., 2007), variants of HRV89 (Reischl et al., 2001; Vlasak et al., 2005a), and HRV8v selected for replication in ICAM-1 negative human cell lines, we became interested in identifying the entry pathway of HS-binding rhinoviruses. In addition, we wanted to examine more closely cellular uptake of HRV14 via ICAM-1. As we had previously demonstrated clathrin-dependent entry of HRV2 (Snyers et al., 2003) we used this virus type as a control in our experiments.

Entry of HRV8v via HS

Absence of co-localization with known markers of clathrin- and caveolin-dependent endocytosis during the early steps of viral uptake clearly showed that HRV8v, when entering via HS, exploits neither of these pathways. Furthermore, when compared to HeLa cells, expression of caveolin was very low in RD cells even though the three HRVs used in this study replicate equally well in these cells (data not shown). These findings were also corroborated by using pharmacological inhibitors. In order to minimize pleiotropic effects, as are common for drugs blocking entry pathways, we used cleavage of eIF4GI at 3 h p.i. as a readout for arrival and translation of the viral RNA in the cytoplasm; viral replication is not required for this process because translation of the incoming RNA produces the proteinase 2A in sufficient amounts for cleavage of this cellular substrate (Glaser and Skern, 2000). Thereby, possible effects of the drugs on later steps, such as RNA synthesis and/or virus release (Gazina et al., 2005) can be excluded. Furthermore, there is no need to maintain the cells in the presence of the drug for extended times, thus cytotoxic potency could be avoided.

Utilizing this experimental setup, we investigated the effects of a number of drugs on viral entry. Out of the panel of compounds amiloride, an inhibitor of Na^+/H^+ exchanger, and dynasore that interferes with dynamin function, abolished cleavage of eIF4GI upon challenging RD cells with HRV8v. A somewhat lesser effect was seen for cytochalasin D, an inhibitor of actin dynamics, and MßCD that sequesters cholesterol, which prevents membrane ruffling and macropinocytosis (Grimmer et al., 2002). Notably amiloride and cytochalasin are well known specific inhibitors of macropinocytosis (West et al., 1989). Inhibition of dynamin is less meaningful as this protein is involved in most entry pathways. When internalized together with FITC-dextran, HRV8v strongly co-localized with this marker of fluid phase uptake again featuring the involvement of macropinocytosis. With respect to the requirement of dynamin in macropinocytosis, HRV8v differs from enterovirus 1 (EV1) (entering via $\alpha_v\beta_v$) (Karjalainen et al., 2008) and from adenovirus 3 (entering via various α_v integrins and CD46) (Amstutz et al., 2008). Uptake of these latter does not require dynamin function and constriction of macropinosomes might be accomplished by C-terminal-binding protein-1/ brefeldinA-ADP ribosylated substrate (CtBP1/BARS). However, as macropinocytosis is dependent on dynamin in HeLa cells (Bonazzi et al., 2005) CtBP1/BARS and dynamin might

replace each other in a cell-type specific manner (Liberali et al., 2008). Finally, a role for flotillin, as indicated from experiments on uptake of proteoglycan-binding ligands (Payne et al., 2007), in HRV8v endocytosis is highly improbable because of only marginal colocalization in immunofluorescence microscopy.

Electron microscopy demonstrated HRV8v binding to shallow invaginations at the plasma membrane. Fluorescence microscopy showed that the virus subsequently accumulated in vesicles larger than those seen in the case of HRV14 and HRV2. Collectively, these compartments can be best described as macropinosomes. As demonstrated by inhibition with bafilomycin, HRV8v requires acidic pH for uncoating and productive infection when entering via HS. This is substantiated by its co-localization with EEA1 at 30 min p.i. (data not shown) thus differing from EV1, which travels to (non-acidic) caveosomes, although not via caveolae (Karjalainen et al., 2008; Liberali et al., 2008). On the other hand, a HS-binding FMDV variant enters via caveolae but it is not entirely clear whether it follows the classical caveolar pathway (O'Donnell et al., 2008). As FMDV depends on low pH for infection, there must be a connection to acidic compartments as well. Indeed, at least glycosylphosphatidylinositol (GPI)-anchored molecules entering via caveolae can reach endosomes (Maxfield and Mayor, 1997). Since most HS is linked to proteins carrying GPI (Filmus et al., 2008), viral arrival in acidic compartments is not unexpected. Collectively, the pathway taken by HRV8v combines characteristics of macropinocytosis with new and specific features indicating the existence of mechanisms slightly differing depending on the cell type.

Entry of HRV14 via ICAM-1

The same colocalization experiments as above carried out with HRV14 gave largely the same results and thus also indicated macropinocytosis. However, the pharmacological inhibitor profile was quite different; out of all drugs only amiloride completely blocked cleavage of eIF4GI, whereas cytochalasin and dynasore showed only marginal effects. These differences between HRV8v and HRV14 might result from the ability of the latter to uncoat at neutral pH by the aid of ICAM-1 and thus not requiring transport to acidic compartments for productive infection; it is possible that HRV14 already uncoats from macropinosomes and that factors

inhibited by cytochalasin and MßCD are needed for further trafficking to acidic compartments. The most remarkable difference in entry of the respective viruses via ICAM-1 and via HS was the requirement of dynamin; dynasore inhibited uptake of HRV8v but did not inhibit uptake of HRV14. This demonstrates two different modes of macropinocytosis. HRV14 uptake into RD-ICAM cells is independent from dynamin (this study) whereas it is dependent on dynamin in HeLa cells (DeTulleo and Kirchhausen, 1998). We confirmed this latter finding, including the cell-type dependence of dynamin requirement (Bonazzi et al., 2005), by our experiments with dynasore; the drug strongly affected HRV14 entry into HeLa cells (data not shown). As mentioned above, evidence is accumulating for cell-type specific effects of this protein on virus uptake. Pertinently, similar observations have been made very recently for Kaposi's sarcoma associated herpesvirus (Raghu et al., 2009).

Electron microscopy of HRV14-infected HeLa cells revealed the virus in coated pits. However, when entering BHK-cells transfected to express human ICAM-1, HRV14 accumulated in long tubular structures with several virions threaded like pearls on a string (Grunert et al., 1997). We obtained almost identical EM images from HRV14 internalized into RD-ICAM cells. These structures are also reminiscent of adenovirus 3 entering HeLa cells via macropinocytosis, as recently shown by use of a number of complementary techniques (Amstutz et al., 2008). This excludes the possibility that the tubules are a consequence of overexpression of ICAM-1 in RD and BHK cells. Similar tubular structures were also observed to contain GPI-anchored proteins (Sabharanjak et al., 2002); however, there is no indication for involvement of lipid rafts in HRV14 uptake into RD-ICAM cells. Interestingly, EV1 entering HeLa cells via integrin by macropinocytosis did not exhibit this "pearl on a string" appearance (Karjalainen et al., 2008). Similar to the absence of reproduction in mouse cells (Harris and Racaniello, 2003), HRV14 failed to replicate in the transfected BHK cells probably because specific host cell factors were absent. Conversely, RD-ICAM cells became productively infected, were lysed, and gave rise to viral progeny. This clearly shows that viral entry via this form of macropinocytosis in tubular structures is productive. Although infection by HRV14 and by other HRVs less stable at low pH is not prevented by bafilomycin because of the "catalytic" activity of ICAM-1, the process is facilitated by low pH (Bayer et al., 1999; Nurani et al., 2003). It is, therefore, also possible that HRV14 is further transferred from the tubular invaginations to acidic vesicles, probably

endosomes, where penetration into the cytosol occurs upon disruption of the vesicular membrane (Schober et al., 1998). Our data agree with results by Muro and colleagues who found that in endothelial cells, multivalent ligands were internalized via ICAM-1 in a clathrin- and caveolin-independent manner (Muro et al., 2003). As pointed out above, inefficient transport to acidic compartments might have a stronger effect on HRV8v as compared to HRV14 since its uncoating definitely depends on low pH whereas the latter virus can be uncoated at neutral pH by ICAM-1 itself.

Whereas internalization of immunobeads coated with anti-ICAM-1 antibodies into endothelial cells was not inhibited by cytochalasin (Muro et al., 2003), HRV14 internalization in RD-ICAM cells was marginally reduced; this is most probably again due to different behavior of the different cell types (endothelial versus rhabdomyosarcoma muscle cells) utilized in these studies.

With respect to dynamin-dependence, entry via ICAM-1 and via integrin, both occurring by macropinocytosis, appear to differ in HeLa cells; the former is inhibited by the dominant-negative dyn^{K44A} mutant (this report and (DeTulleo and Kirchhausen, 1998)) whereas the latter is not (Karjalainen et al., 2008). However, it was shown previously that the degree of the inhibition substantially depends on the cell type (Amstutz et al., 2008). Therefore, there might be subtle differences in macropinocytosis of HRV14 via ICAM-1 in RD-ICAM cells and of Ad3 via α_v integrin/CD46 in HeLa cells despite occurring through morphologically identical tubes in the form of "pearls on a string".

Entry of HRV2

Although HRV2 was merely used as a control for clathrin-dependent endocytosis, we made some new findings with the pharmacological inhibitors. The endocytosis-blocking effect of chlorpromazine and dynasore was expected, and the inhibition by cytochalasin can be explained by the well-known involvement of actin in clathrin-dependent endocytosis (Lamaze et al., 1997). However, we were surprised to find a complete inhibition of eIF4G1 cleavage by amiloride. It can be excluded that this drug affects entry because immunofluorescence microscopy clearly showed that virus entered the cells in its presence. For coxsackievirus B3

and for HRV2, inhibition of RNA replication and of virus release, respectively, by amiloride has been demonstrated (Gazina et al., 2005; Harrison et al., 2008). Our data rather point to inhibition of RNA release into the cytosol or to RNA replication although the latter most probably does not play a role in our cleavage assay. We also found lack of inhibition by MßCD, which contrasts to earlier findings (Snyers et al., 2003). In the absence of additional experimentation we tentatively explain this by the difference in cell type used (i.e. HeLa cells in the previous and RD cells in the present work).

Poliovirus entry into HeLa cells occurs via a caveolin- and dynamin-independent pathway, whereas entry into brain microvascular endothelial cells depends on both components (Coyne et al., 2007). We have now demonstrated a similar situation for rhinoviruses. Different representatives of the same virus species use different receptors and enter via different pathways. Even the same virus could enter into different cell types via macropinocytosis either in a dynamin-dependent or independent manner. This is an interesting example for the versatility of viruses to exploit whatever suitable way to breach cellular membranes for infection. By using three virus types belonging to the same genus of *rhinoviridae* we also demonstrate the existence of distinct entry pathways with different final destinations in identical cell types.

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1. Khan, A.G., Pichler, J., Rosemann, A., and Blaas, D. (2007). Human rhinovirus type 54 infection via heparin sulfate is less efficient and strictly dependent on low endosomal pH. J Virol *81*, 4625-4632

2. Khan, A.G., Pickl-Herk, A., Marlovits, T., Fuchs, R., and Blaas, D. (2009). Macropinocytosis and Clathrin-dependent Uptake of Human Rhinoviruses: Usage of Different Receptors Leads to Different Entry Pathways (submitted)

3. Berka, U., Khan, A., Blaas, D., and Fuchs, R. (2009). HRV2 Uncoating at the Plasma Membrane is not Affected by a pH Gradient but by the Membrane Potential. J. Virol. doi:10.1128/JVI.01739-08

4. Oral presentation at Europic 2006 in Ivalo Finland

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Abdul Ghafoor Khan

Human Rhinovirus Type 54 Infection via Heparan Sulfate Is Less Efficient and Strictly Dependent on Low Endosomal pH[▽]

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K-type major-group human rhinoviruses (HRVs) (including HRV54) share a prominent lysine residue in the HI surface loop of VP1 with all minor-group HRVs. Despite the presence of this residue, they cannot use members of the low-density lipoprotein receptor family for productive infection. Reexamining all K-type viruses for receptor usage, we noticed that HRV54 is able to replicate in RD cells that lack the major-group receptor intercellular adhesion molecule 1 (ICAM-1). By using receptor blocking assays, inhibition of sulfation, enzymatic digestion, and proteoglycan-deficient cell lines, we show here that wild-type HRV54, without any adaptation, uses heparan sulfate (HS) proteoglycan as an alternate receptor. However, infection via HS is less efficient than infection via ICAM-1. Moreover, HRV54 has an acid lability profile similar to that of the minor-group virus HRV2. In ICAM-1-deficient cells its replication is completely blocked by the H^+-ATPase inhibitor bafilomycin A1, whereas in ICAM-1-expressing cells it replicates in the presence of the drug. Thus, use of a "noncatalytic" receptor requires the virus to be highly unstable at low pH.

Human rhinoviruses (HRVs), the most important pathogens in the origin of the common cold, are small, icosahedral, nonenveloped, plus-stranded RNA viruses belonging to the *Picornaviridae* family (27). HRVs circulate as 99 serotypes; based on comparison of the amino acid sequences of their capsid protein VP1, they were phylogenetically classified as subgenera HRV-A and HRV-B (15, 16). Independent from this classification, they are also divided into minor and major receptor groups. The minor group comprises 12 serotypes that use members of the low-density lipoprotein receptor (LDLR) family for cell entry, whereas major-group viruses attach to intercellular adhesion molecule-1 (ICAM-1) for infection (36). The LDLR family includes the LDLR, the very-low-density lipoprotein receptor (VLDLR), and LDLR-related protein 1, which are all recognized by minor-group HRVs, and many other proteins with similar architecture that probably do not function as viral receptors (20). Major-group HRVs of the two subgenera possess two respective sequence patches involved in ICAM-1 recognition (14); minor-group HRVs have only a strictly conserved lysine residue in the HI surface loop of VP1 that is necessary but not sufficient for receptor binding (36). Thus, the principles underlying receptor discrimination are still poorly understood.

There is also a species-specific discrimination in virus-receptor interaction. For example, HRV1A binds more strongly to mouse LDLR and very weakly to the human homologue, whereas HRV2 can bind equally well to both (9, 25), albeit that these serotypes belong to the minor receptor group and are of subgenus A. LDLRs presumably act only as vehicles to transport the virus across the plasma membrane within clathrin-coated vesicles; upon arrival in endosomes, the low-pH milieu destabilizes the viral capsid and the RNA is released. Minor-group viruses, at least HRV2, strictly depend on the low endosomal pH for uncoating (18, 22). ICAM-1, on the other hand, not only acts as a receptor for binding and cell entry but also facilitates uncoating (7).

As mentioned above, a single lysine residue at the capsid surface is conserved in all minor-group viruses. Interestingly, there are 9 "K-type" major-group HRVs (HRV8, -18, -24, -40, -54, -56, -58, -85, and -98; all are subgenus A) that also possess a lysine at the same position; this suggested that they might be able to use LDLR as well. However, all of these K-type viruses failed to infect HeLa cells in the presence of the ICAM-1-blocking monoclonal antibody (MAb) R6.5 (36). While screening these serotypes for their ability to infect human rhabdomyosarcoma (RD) cells that lack ICAM-1 expression (28), we were surprised to find that HRV54 is able to infect this cell line. We also noticed that increasing the 50% tissue culture infective dose ($TCID_{50}$)/well (of a 96-well plate) from 10^3, as used in the previous study (36), to 10^5 resulted in infection of HeLa-H1 cells whose ICAM-1 was blocked with MAb R6.5. However, as shown previously, infection was not affected by MBP-V33333, a recombinant soluble concatemer of VLDLR repeat 3, which is a potent inhibitor of minor-group viruses (34).

Heparan sulfate (HS) is ubiquitously expressed at the surfaces of mammalian cells. The natural functions of this glycosaminoglycan include cell adhesion, migration, proliferation, and differentiation; it also binds a number of signaling molecules and many other ligands (33). Recently, it has been found that many viruses can use cell surface HS proteoglycans for attachment and entry. Among the family *Picornaviridae*, foot-and-mouth disease virus, swine vesicular disease virus, coxsackievirus B3, Theiler's murine encephalomyelitis virus, some echoviruses, and variants of HRV89 have been shown to in-

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teract with HS for cell attachment and entry, especially in the absence of their classic receptors (4, 6, 11, 23, 35, 37). Having seen that HRV54 can infect the ICAM-1-negative RD cells but is not inhibited by the recombinant VLDLR derivative, we set out to identify the receptor used in addition to ICAM-1. We show here that it is HS proteoglycan (HSPG). In contrast to the previous report on HRV89 variants using HS as result of a tedious adaptation for growth in HEp-2 cells that express low levels of ICAM-1, HRV54 already possesses this property as the wild type (wt) without prior adaptation.

Employing the specific H^+-ATPase inhibitor bafilomycin A1 (Baf), we present evidence that use of a "noncatalytic" receptor that does not facilitate uncoating, such as HS, requires the virus to be highly acid sensitive. Our data illustrate the great plasticity of these viruses with respect to receptor usage and once again demonstrate that the use of a given receptor calls for particular physicochemical properties of the virus.

MATERIALS AND METHODS

Cells, viruses, and chemicals. HRV2 was originally obtained from the American Type Culture Collection (ATCC) and was propagated in HeLa-H1 cells. HRV54 was from the National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands; it was passaged twice in HeLa-OHIO cells in the Enterovirus Laboratory in Helsinki, Finland, and kindly given to us. It was further passaged six times in HeLa-H1 cells in our laboratory. All experiments with HRV54 were carried out with an isolate obtained from a single plaque whose identity was confirmed by neutralization with serotype-specific guinea pig antiserum from the ATCC. Human RD wt and RD-ICAM cells (stably transfected to express human ICAM-1) (13) were a kind gift from Darren R. Shafren, University of Newcastle, New South Wales, Australia. The wt cells do not express any ICAM-1, as verified by fluorescence-activated cell sorter analysis. These cells were grown in Dulbecco's modified Eagle medium supplemented with fetal calf serum (10% in growth medium and 2% in infection medium, which also contained 30 mM $MgCl_2$), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. CHO-K1 cells and CHO mutants (pgsA-745 and pgsD-677) deficient in proteoglycan biosynthesis were obtained from the ATCC. These cells were grown in Ham's F-12 medium supplemented with 5% FCS, L-glutamine, and antibiotics as described above. Heparinase 1 (H2519) was purchased from Sigma-Aldrich GmbH, Vienna, Austria, and Na-chlorate was from Alfa Aesar GmbH, Germany.

Infection inhibition assays. RD cells were grown in 96-well microtiter plates to 80% confluence. Virus (at 50 $TCID_{50}$/cell) was added in the presence or absence of twofold serial dilutions of heparin or HS in the infection medium. Plates were incubated at 34°C with 5% CO_2 until cells in the controls (without glycans) showed more than 90% cytopathic effect; this was generally at 36 h post infection (p.i.). Cells were fixed with paraformaldehyde (4% in phosphate-buffered saline [PBS]), washed with PBS, and stained with 0.1% crystal violet. After washing with water, the stain was eluted with methanol. Cell damage was quantified with respect to the intensity of the stain retained by living cells in a plate reader at 630 nm. Cell survival in the presence of inhibitors was calculated by setting mock-infected cells to 100% survival and cells infected without inhibitor to 0% survival (12).

Assay for double receptor specificity. RD-ICAM cells were seeded in 96-well plates and were infected with HRV54 at 10 $TCID_{50}$/cell in the presence or absence of the ICAM-1-blocking MAb R6.5 (24) with or without heparin. Cells were incubated for 1 h with 100 μg/ml of R6.5, whereas virus was incubated with 2 mg/ml of heparin for 1 h at 34°C prior to addition to the cell monolayer. After 24 h at 34°C the cells were fixed, and cell damage was monitored as described above. For binding assays, cells grown in 12-well plates were preincubated with MAb R6.5 and challenged with 12,000 cpm of ^{35}S-labeled HRV54 (18) in the presence or absence of heparin. After incubation for 1 h at 34°C, the cells were washed, and cell-bound radioactivity was determined by liquid scintillation counting.

Cell binding inhibition assays. RD cells were grown in 12-well plates and washed with Hanks buffered salt solution (HBSS). ^{35}S-labeled HRV54 at ~12,000 cpm in HBSS was added in the presence or absence of the glycosaminoglycan, and the cells were incubated for 60 min at 34°C with gentle rocking,

washed with HBSS to remove unbound virus, and detached with trypsin-EDTA. Cell-associated radioactivity was determined as described above.

Incorporation of sulfates into the proteoglycans was inhibited with chlorate as described previously (35). Briefly, RD cells were grown in 24-well plates in medium supplemented with 50 mM $NaClO_3$ for 3 days, and binding of radiolabeled virus (in the presence or absence of 2 mg/ml of heparin, where mentioned) was assayed as described above. For digestion of proteoglycans, RD cells grown in 24-well plates to 80% confluence were washed with PBS and incubated with 4, 2, and 1 U/ml of heparinase 1 in HBSS for 2 h at 37°C. Digested material was removed by washing with ice-cold HBSS and virus binding at 4°C was determined.

To determine attachment to proteoglycan-deficient cells, CHO-K1 (wt), pgsA-745, and pgsD-677 cells were seeded in 24-well plates and grown to 90% confluence. Cells were washed with HBSS, challenged with ^{35}S-labeled virus at 16,000 cpm, and incubated at 34°C for 1 h with gentle rocking. Cell-associated radioactivity and radioactivity in the supernatant were determined as above. The percent virus binding was calculated.

Acid sensitivity of the virus. Virus at 10^7 $TCID_{50}$ was incubated with 0.5 M sodium acetate buffer at pH 7.0, 6.5, 6.0, 5.6, 5.2, 4.8, and 4.4 for 30 min at room temperature and neutralized with 0.5 M Na_3PO_4. Infectivity was determined by end point dilution assay.

Effect of Baf on virus uncoating. RD cells grown in 96-well plates were incubated with 200 nM of Baf (Alexis Biochemicals, Switzerland) for 1 h at 37°C. Virus at 100 $TCID_{50}$/cell in infection medium containing 100 nM Baf was added to the cells, and incubation was continued for 24 h at 34°C. To investigate the role of ICAM-1 in virus uncoating, RD-ICAM cells were used in parallel. As a control, to block the ICAM-1, cells were also incubated with 100 μg/ml of R6.5 for 1 h prior to virus addition. Infection was evaluated as described before.

To test whether viral de novo synthesis occurs in the presence of Baf, RD and RD-ICAM cells were grown in 24-well plates to 80% confluence. Cells were washed with PBS and incubated with or without 200 nM Baf in 200 μl of infection medium for 1 h at 37°C. Virus at 10^6 $TCID_{50}$ in 200 μl infection medium (about 1 $TCID_{50}$/cell) was added, and incubation was continued for 1 h at 34°C. Cells were washed twice with ice-cold PBS, and 200 μl of infection medium with and without 100 nM Baf was added. Incubation was continued for a further 0, 2, 12, and 23 h. Cells were then lysed by three freeze-thaw cycles. Cell debris was pelleted, and the virus titer in the supernatant was determined.

RESULTS

HRV54 infection is inhibited by soluble glycosaminoglycans. Recently, several representatives of the *Picornaviridae* family have been shown to use HSPG as an alternate receptor. Moreover, the rhinovirus serotype HRV89 was adapted to grow in cells devoid of ICAM-1, and these variants also use HSPG as a receptor (35). After having noticed the ability of HRV54 to infect ICAM-1-negative RD cells and the lack of inhibition by the VLDLR derivative MBP-V33333 (data not shown), we asked whether this serotype might also use HSPG for infection. We thus tested glycosaminoglycans for their ability to inhibit infection. Cells were challenged with HRV54 in the presence of serial twofold dilutions of heparin and HS, and cell survival was evaluated. As shown in Fig. 1, both glycosaminoglycans reduced the cytopathic effect of the virus. The more strongly sulfated heparin showed more inhibition than HS. The former, at 1 mg/ml, was sufficient to completely protect the cells. For HS, complete inhibition was observed at 2 mg/ml, with almost no inhibition below 0.5 mg/ml.

To exclude a role of heparin other than competing with cell surface glycosaminoglycans, we carried out a cell protection experiment in three different ways (4): (i) virus was incubated with heparin for 30 min at 34°C, and the mixture was then transferred onto the cells; (ii) cells were incubated with virus at 4°C for 30 min, unbound virus was removed by washing, and heparin was added; and (iii) cells were incubated with heparin for 30 min at 37°C and washed, and virus was added. As
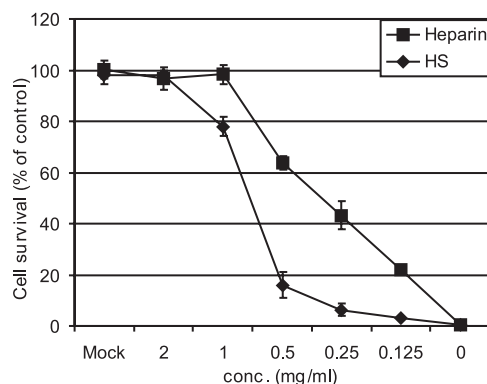
FIG. 1. Heparin and HS inhibit cytopathic effect upon infection of RD cells with HRV54 in a concentration-dependent manner. RD cells grown in 96-well plates were infected with HRV54 (50 $TCID_{50}$/cell) in the presence of the glycans at the concentrations indicated. Plates were incubated at 34°C until cells in the control (no glycan added) showed >90% damage. Cells were fixed and stained with crystal violet, and the dye was eluted with methanol and quantified in a plate reader. Survival of mock-infected cells was set to 100%, and that in the absence of the glycans was set to 0%. Values are means from three parallel experiments ± standard deviations.

expected, we observed maximum inhibition of infection when the virus was incubated with heparin prior to challenging the cells. We also observed an inhibition of up to 40% when heparin was added after virus attachment at 4°C but only at the highest concentration (1 mg/ml). Possibly, already attached virus could be eluted from the cell surface by the heparin. Finally, incubation of the cells with heparin did not modify infection to any significant extent (data not shown). These results clearly show that the soluble derivatives of the cell surface proteoglycan physically interact with the virus and thus block its binding sites for the cell surface receptors.

Heparin and HS suppress HRV54 binding to RD cells. To obtain additional evidence for HRV54 using HS as a receptor, we carried out binding inhibition assays using ^{35}S-labeled virus. RD cells were incubated with radiolabeled virus in the presence or absence of the glycosaminoglycans at 34°C for 60 min. Unbound virus was removed, and cell-associated radioactivity was determined. Heparin showed a concentration-dependent inhibition of binding (Fig. 2A). We also tested HS, chondroitin sulfate (CS), and dermatan sulfate (DS) for their ability to compete with the natural receptor. Only heparin and HS reduced HRV54 binding to the background level, while DS showed some weak inhibition and the effect of CS was only marginal (Fig. 2B). As these proteoglycans differ in monosaccharide moiety and degree of sulfation, these results indicate specificity for particular sulfation sites and/or oligosaccharide structures. It is likely that the interaction preferentially relies on the high concentration of sulfate groups present in HS. At very high, physiologically irrelevant concentrations, DS and CS possibly also inhibit virus binding.

Sulfation is required for virus binding. Further evidence for the involvement of sulfated proteoglycans was obtained by the suppression of sulfate incorporation into proteoglycans. RD cells were grown for 3 days in medium with and without $NaClO_3$, washed, and incubated with radiolabeled virus. Unbound virus was removed, and cell-associated radioactivity was
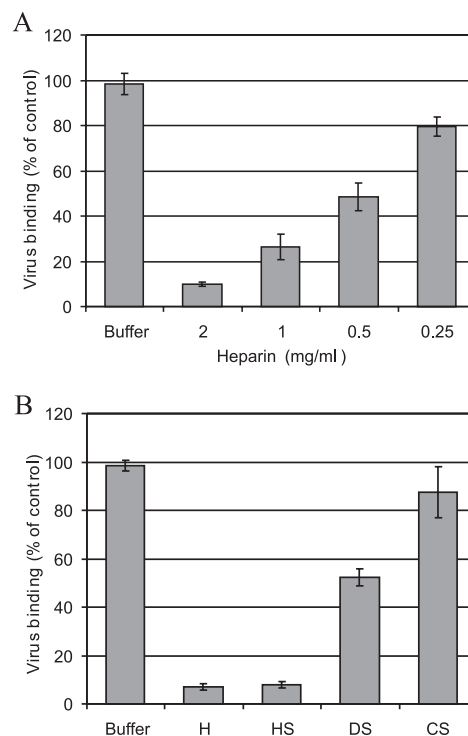

FIG. 2. Heparin and HS inhibit HRV54 binding to RD cells. Cells were grown in 12-well plates, washed, and challenged with ^{35}S-labeled HRV54 at ~16,000 cpm in 0.4 ml HBSS with gentle rocking for 60 min at 34°C in the presence of glycans as indicated. Inhibition by heparin (H) was assessed at concentrations of between 0.25 and 2 mg/ml (A), and inhibition by the respective glycans was assessed at 2 mg/ml (B). Cells were washed to remove unbound virus, and cell-associated radioactivity was measured by liquid scintillation counting. Virus binding in the absence of the glycans (buffer) was set to 100%. Values are means from three independent experiments ± standard deviations.

quantified. As shown in Fig. 3, HRV54 binding to cells grown in the presence of chlorate was reduced by 65% compared to that to control cells. However, binding further diminished to about 10% in the presence of heparin. This is probably due
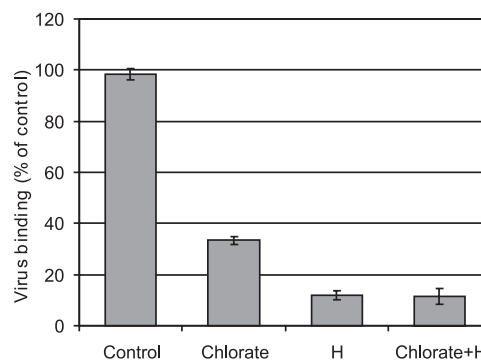

FIG. 3. RD cells grown in medium containing 50 mM $NaClO_3$ exhibit reduced HRV54 binding. Cells were grown in the presence and absence (control) of 50 mM $NaClO_3$ for 3 days, washed, and incubated with virus at ~16,000 cpm for 60 min at 34°C. Unbound virus was washed away, and cell-associated radioactivity was quantified. Binding is shown as a percentage of the control value (cells grown in the absence of chlorate and without addition of heparin [H]). Values are means from three independent experiments ± standard deviations.
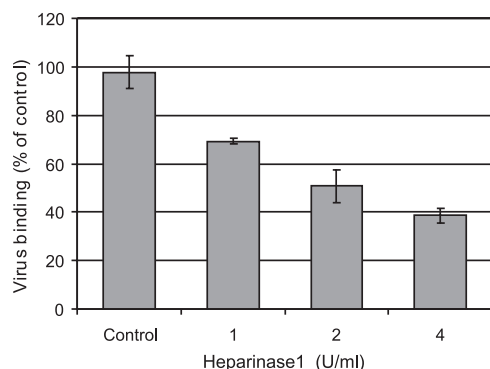
FIG. 4. Heparinase 1 treatment of RD cells decreases HRV54 binding in a concentration-dependent manner. Cells grown in 24-well plates were washed with HBSS and incubated with the indicated concentrations of heparinase 1 for 2 h at 37°C. Digested material was removed with ice-cold buffer, cells were challenged with 16,000 cpm of HRV54, and incubation was continued for 1 h at 4°C. Unbound virus was washed away, cells were trypsinized, and cell-associated radioactivity was determined. The value for virus bound to nontreated cells (control) was set to 100%. Values are means from three independent experiments ± standard deviations.
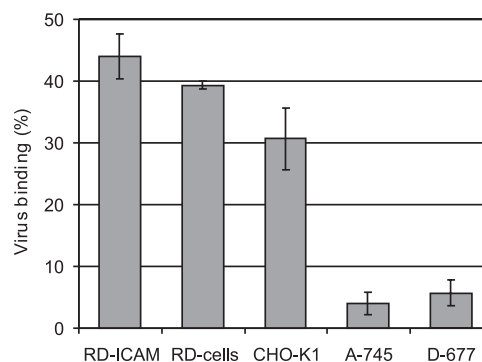

FIG. 5. CHO mutant cells deficient in proteoglycan synthesis show only background binding of HRV54. Cells were seeded in 24-well plates, grown to 90% confluence, and washed with HBSS. ^{35}S-labeled HRV54 at 12,000 cpm was, added and the plates were incubated at 34°C for 60 min. Unbound virus was washed away. Cells were trypsinized, and cell-associated radioactivity and radioactivity in the supernatant were quantified. The percentage of bound virus with respect to total input virus is shown. Values are means from three independent experiments ± standard deviations.

to incomplete suppression of sulfate incorporation by the chlorate.

Heparinase 1 treatment of RD cells reduces HRV54 attachment. In order to obtain direct evidence for HRV54 binding to HSPG, HS was enzymatically removed. RD cells grown in 24-well plates were incubated with increasing concentrations of heparinase 1 for 2 h at 37°C and washed to remove digested material, and radiolabeled virus was added. After incubation for 60 min at 4°C, unbound virus was removed, and cell-associated radioactivity was determined. As seen in Fig. 4, virus binding was significantly decreased with increasing concentration of the enzyme used to digest cell surface-exposed HS.

CHO mutant cells deficient in proteoglycan synthesis fail to bind HRV54. To further confirm the involvement of cell surface HSPG in virus binding, we made use of CHO mutant cells deficient in proteoglycan synthesis. These cells have been extensively used for the investigation of virus-HSPG interactions (17). CHO pgsA-745 cells are deficient in xylosyltransferase synthesis and are unable to produce any glycosaminoglycans, and pgsD-677 cells are doubly deficient in N-acetylglucosaminyltransferase and glucuronyltransferase and fail to synthesize HS but produce threefold-higher levels of CS than the wt. These cells, along with wt CHO-K1, were challenged with ^{35}S-labeled HRV54, and bound virus was determined as described above. A significant difference in binding to the different cell lines was evident (Fig. 5). RD-ICAM cells possessing both receptors, ICAM-1 and HS, strongly bound HRV54, followed by the cells devoid of ICAM-1. wt CHO-K1 cells also bound HRV54 significantly (about 77% compared to RD cells), whereas the mutant cell lines showed only background binding. These results again confirm that HRV54 binds to cell surface HSPG.

HRV54 has double receptor specificity. Human ICAM-1 is the receptor for major-group viruses. This receptor not only is responsible for cell attachment but also facilitates virus uncoating. Since HS is not expected to possess any catalytic activity, we asked whether HRV54 can use both receptors indepen-

dently. RD-ICAM cells were preincubated with R6.5. This is an ICAM-1-specific MAb that blocks the virus binding site as exemplified with HRV14 (29). On the other hand, virus was incubated with heparin. Infection was then monitored in the presence of MAb R6.5 alone or in combination with heparin. After 24 h, cell survival was quantified (Fig. 6A). As expected, neither MAb R6.5 nor heparin alone prevented cell damage, while in combination almost 100% of the cells remained alive. These results clearly show that HRV54 can use either of the receptors independently for productive infection. The same experimental setup was used to monitor virus binding. RD-ICAM cells were preincubated with MAb R6.5 and challenged with radiolabeled HRV54 in the presence or absence of heparin as described above. Surprisingly, MAb R6.5 failed to inhibit virus binding, whereas heparin suppressed attachment by 40%. This indirectly suggests that the remaining 60% binding is due to ICAM-1. The lack of binding inhibition by MAb R6.5 indicates that the cellular HS provides many more attachment sites than ICAM-1; with the former receptor blocked, the limited ICAM-1 binding sites become quickly saturated, and only 60% of input virus can be accommodated. Virus binding was reduced to background values when both inhibitors were used together (Fig. 6B). This excludes the existence of a third receptor.

HRV54 is highly acid labile. A prime characteristic of HRVs is their acid lability, which has been widely used to differentiate them from enteroviruses (30). HRVs usually become inactivated at a pH of <3 (10). However, some serotypes (e.g., HRV2) undergo structural changes associated with uncoating already at a pH of ≤5.6, and most probably all minor-group viruses are uncoated at a pH of around 5.5, which prevails in late endosomes (8, 18). On the other hand, the catalytic activity of ICAM-1 allows infection of HRV14, HRV3, and HRV89 even in the presence of endosomotropic agents like Baf that increase the endosomal pH to neutrality (1, 19, 35). The ability of HRV54 to infect RD cells lacking ICAM-1 might indicate that it has to be highly acid labile, allowing the low endosomal pH alone to trigger uncoating. We thus determined virus in-
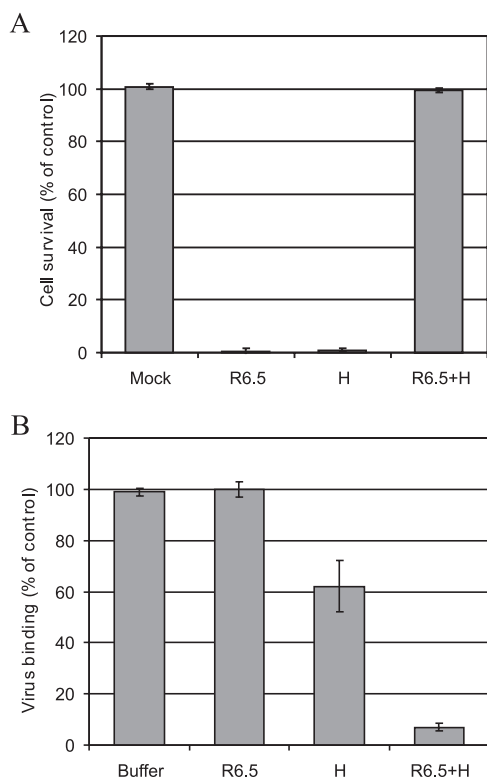
A

B

FIG. 6. HRV54 can use ICAM-1 and HS independently for binding and productive infection. (A) Cells grown in 96-well plates were incubated with MAb R6.5 for 1 h at 37°C to block ICAM-1. To block heparin binding sites, virus was incubated for 30 min at 34°C with heparin (H). Cells were challenged, and after 24 h at 34°C, cell survival was determined as described for Fig. 1. Note that only the combination of MAb R6.5 and heparin completely protects RD-ICAM cells from viral damage. (B) Cells grown in 12-well plates were treated as described above and challenged with about 15,000 cpm of radiolabeled HRV54 with or without heparin for 60 min at 34°C. Binding in plain buffer (in the absence of R6.5 and heparin) was set to 100%. Values are means from three independent experiments ± standard deviations.
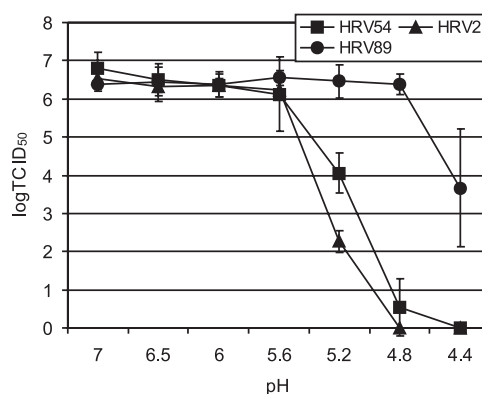

FIG. 7. HRV54 is more sensitive to low pH than HRV89, another major-group virus. HRVs were incubated in buffer at the pH indicated for 30 min at room temperature. The solutions were neutralized, and virus infectivity was determined. HRV2 and HRV89 were used as representatives for each receptor group. Values are means from three independent experiments ± standard deviations.

activation as a function of the pH. HRVs at 10^7 $TCID_{50}$ were incubated in sodium acetate buffer adjusted to pH 7, 6.5, 6.0, 5.6, 5.2, 4.8, and 4.4 for 30 min at room temperature. After reneutralization with phosphate buffer, the infectivity was determined. HRV2 and HRV89 were used as controls representing each receptor group. As depicted in Fig. 7, HRV54 and HRV2 were inactivated at below pH 5.6, while HRV89 remained infective even at pH 4.4, although at substantially reduced titer. The pH thresholds for inactivation, as determined in this experimental setup, are all somewhat below the pH encountered in late endosomes, the site of uncoating; nevertheless, they indicate a similar acid lability of HRV54 and HRV2. This suggests that usage of a receptor different from ICAM-1 requires the virus to be unstable at a pH that prevails in the endosomal system. This correlates well with the previous data on the HRV89 mutants; acquiring affinity for HS went hand in hand with the virus becoming less acid stable than the wt (35).

HRV54 uncoating in the absence of ICAM-1 depends on low endosomal pH. Baf is well established in investigating the role of the low endosomal pH in virus uncoating. This drug inhibits

vesicular H^+-ATPases and has been widely employed in investigations of the influence of the endosomal pH on HRV2 and HRV14 infection (1, 22, 35). For example, HRV2 infection is completely blocked by Baf, while infection with HRV14 proceeds, albeit with lower efficiency (1, 19). This is in agreement with the in vitro experiments indicating that ICAM-1-catalyzed uncoating occurs at neutral pH (7). We thus conducted experiments to investigate the pH dependency of HRV54 infection. RD cells were preincubated with Baf and infected with HRV54, and cell damage was monitored at 24 h p.i. As shown in Fig. 8A, virtually 100% of the cells survived in the presence of the drug; thus, in RD cells, HRV54 behaves as HRV2 does with respect to strict dependence on the low endosomal pH for uncoating. When the same experiment was carried out using RD-ICAM cells, substantial cell damage occurred in the presence of Baf, making it clear that the low pH is not required for HRV54 infection if ICAM-1 is present (Fig. 8B). However, when ICAM-1 was blocked with MAb R6.5, the RD-ICAM cells behaved like wt RD cells.

We further confirmed the dependency of the virus uncoating on the low endosomal pH by determining de novo virus production in the presence or absence of the drug. Virus replication took place in the presence of Baf in RD-ICAM cells (Fig. 9A) but not in wt RD cells (Fig. 9B). These results further support the strict requirement of HRV54 for a low-pH environment for uncoating and infection when using HS as a receptor in the absence of ICAM-1.

DISCUSSION

Although phylogenetically very similar and causing the same disease, the many rhinovirus serotypes use two structurally and functionally unrelated receptors for infection and follow distinct pathways for uncoating (2, 26). In particular, a single lysine residue in the HI loop of VP1 of minor-group HRVs is essential in the interaction with members of the LDLR family (34) that bind close to the fivefold-symmetry axis, whereas ICAM-1 binds within the viral canyon of major-group HRVs (21). This lysine residue is strictly conserved in all 12 minor-
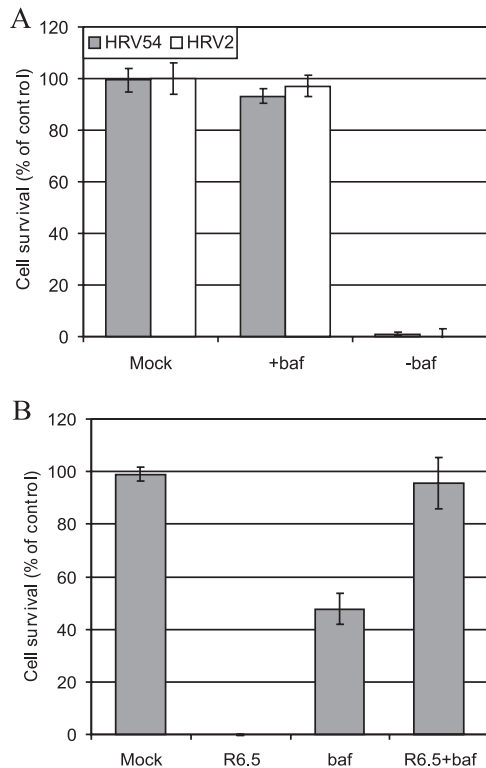
FIG. 8. In the absence of ICAM-1, HRV54 infection depends on the low endosomal pH. RD cells (A) and RD-ICAM cells (B) were preincubated with Baf and infected with HRV54 (and also with HRV2 in the case of RD cells). Where indicated, MAb R6.5 was also present during the entire experiment. Cell survival was monitored after 24 h as described for Fig. 1. Values are means from three independent experiments ± standard deviations.
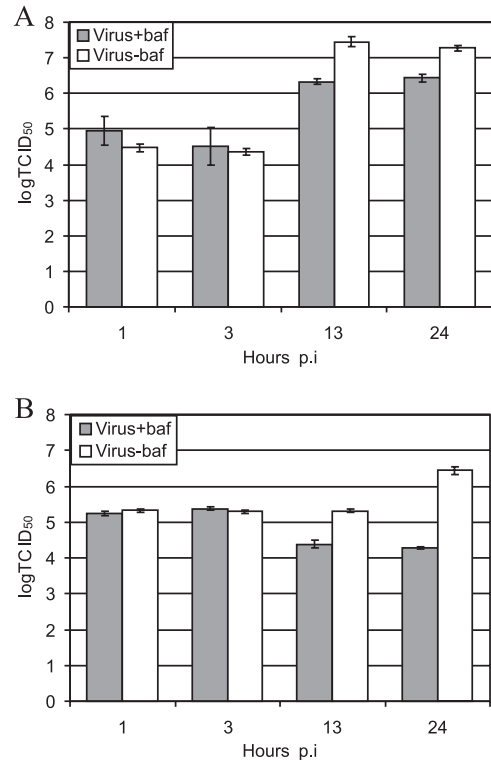

FIG. 9. Effect of Baf on viral replication in RD-ICAM (A) and RD (B) cells. Cells were preincubated with or without Baf and infected with HRV54. After the incubation times indicated, the titer of the virus was determined. Note that the decrease in virus titer in the presence of Baf at 13 and 24 h p.i. (B) is due to degradation, which is not counterbalanced by de novo viral synthesis. Values are means from three parallel experiments ± standard deviations.

group HRVs and is also present in nine serotypes of the major group of rhinoviruses, yet neither of these latter groups is able to use LDLRs for infection. This indicates that the lysine is necessary but not sufficient (36). Reexamination of the receptor specificity of these K-type HRVs at a high multiplicity of infection (50 $TCID_{50}$/cell) revealed, to our surprise, that HRV54 was able to kill RD cells that lack ICAM-1 expression. Since infection by this serotype was not inhibited by MBP-V33333, a recombinant concatemer of repeat 3 of VLDLR that strongly neutralizes all minor-group HRVs (34, 36), involvement of LDLRs in cell attachment was excluded. We thus investigated cell surface HS as a likely candidate receptor. Indeed, heparin as well as HS strongly inhibited HRV54-induced cytopathic effect in RD cells, but with different efficiencies. The more sulfated heparin completely protected the cells already at concentrations of ≥1 mg/ml. This compares well with HS-binding echoviruses, which require between 125 μg/ml and 2 mg/ml of heparin to completely prevent infection (6). The involvement of HS in virus binding was further supported by treatment of the cells with heparinase 1 and the use of CHO mutants with defects in glycan synthesis; virus attachment was drastically reduced upon enzymatic removal of HS, and CHO pgsA-745 and pgsD-677 cells showed only background binding.

The interaction between HRV54 and HS is relatively specific, since DS and CS were only marginally effective in preventing virus binding. Furthermore, sulfate modification of the glycan is required, since growth of the cells in the presence of chlorate substantially reduced attachment of HRV54.

The type of interaction between HS and various ligands, including viruses, is mostly of an ionic nature. The binding site is often formed by basic residues that are far from each other in the sequence but come close together in the three-dimensional structure. However, the HS binding motifs BBXB and BBBXXB (with B being a basic residue and X any other) have also been characterized in some cases (32). Scanning of VP1 of HRV54, the only capsid protein with an available sequence, using ScanProsite (http://www.expasy.ch/cgi-bin/prosite/PSScan.cgi) revealed the presence of such a pattern (HHFK) at the BC loop. Interestingly, a similar pattern was also found in HRV62, -65, -83, and -98 at a comparable position. Since the three-dimensional structures of all these serotypes are not available, it is not known whether this motif is sufficiently accessible for binding. Even more, the presence of an HS binding motif does not necessarily mean that it is being used (5), and there are some viruses which lack such a sequence pattern altogether and yet bind HS (4, 24, 35).

Preincubation of HRV54 with heparin (to block its heparin binding sites) together with blocking of ICAM-1 on the cell surface with MAb R6.5 completely prevented infection, indicating that receptor usage is limited to ICAM-1 and HS. This makes the involvement of an additional receptor, including

LDLRs, unlikely. Interestingly, MAb R6.5 did not appreciably diminish binding of radiolabeled virus to RD-ICAM cells, whereas heparin reduced virus attachment by about 40%. Apparently, the ICAM-1 molecules are limiting in virus binding whereas HS molecules are not.

ICAM-1 has a catalytic ability facilitating uncoating, presumably by stabilizing an intermediate conformation of the capsid. Furthermore, RNA release also occurs in the presence of lysosomotropic agents and drugs that neutralize the endosomal pH (19). Since LDLRs lack such an activity, the minorgroup virus HRV2, and most probably all members of the minor group, are strictly dependent on the low-pH environment for uncoating. We thus asked how HVR54 is uncoated in the absence of ICAM-1 and determined its pH stability. Comparison with HRV2 and the major-group HRV89 revealed that it exhibits a low acid stability similar to that of HRV2. Is the conserved lysine in the HI loop of VP1 responsible for this property? To address this question we also tested the pH sensitivities of some of the other K-type HRVs. Interestingly, we observed that the other major-group K-type viruses tested (HRV8, -18, and -24) were substantially more stable at low pH (data not shown). This makes a contribution of the lysine in pH lability unlikely. The threshold pH for HRV2 inactivation was somewhat lower than the one previously determined (8), which might be due to different experimental setups. Nevertheless, it is evident that HRV2 and HRV54 exhibit similar acid sensitivity profiles. Accordingly, like in the case of HRV2, infection by HRV54 was indeed completely blocked by the vesicular H$^+$-ATPase inhibitor Baf in the absence of ICAM-1; a slight decrease in viral titer at 13 and 24 h p.i. is most probably due to inactivation of the input virus (Fig. 9B).

A number of enteroviruses have been shown to use HS as an alternate receptor for cell entry; since most of them normally attach to members of the immunoglobulin superfamily that bind within the viral canyon and aid in uncoating, it is not clear how the RNAs of these acid-stable viruses become released within the cell. Are there additional factors catalyzing uncoating? It would be interesting to examine the HS-binding enteroviruses for their pH stability. They might be more labile than those that exclusively bind ICAM-1, the poliovirus receptor, or the coxsackie-adenovirus receptor.

Finally, we compared the efficiencies of infection via the two different receptors. The virus yield was consistently by about 1 order of magnitude lower when ICAM-1-negative cells were infected, and appreciable viral de novo synthesis was clearly seen only at 24 h p.i., whereas it was virtually finished within 13 h in RD-ICAM cells. This went hand in hand with cell death; no RD-ICAM cells were left at 13 h, but some few intact RD cells were still found at 24 h p.i. This fact can be tentatively explained by assuming that the uncoating either is less efficient or occurs at an unfavorable site within the endocytic pathway when ICAM-1 is absent.

In summary, our results demonstrate that HRV54, a majorgroup HRV, is able to use HS in addition to ICAM-1 for productive infection. In contrast to HRV89, which required extensive adaptation (32 blind passages in HEp-2 cells that express ICAM-1 only at a very low level, each followed by a boost in HeLa cells) to acquire HS binding (24, 35), this property seems to be intrinsic to wt HRV54. Some other picornaviruses have been shown to acquire HS binding on tissue cul-

ture propagation. Since natural HRV54 isolates were not available for comparison, we cannot exclude that a few passages in tissue culture suffice for adaptation. However, the lack of HS binding by HRV2 and HRV14, which have been serially passaged many times in our laboratory, makes this unlikely and demonstrates that the phenomenon is not a general one.

Although there is abundant HS on the cell surface and attachment appears to be difficult to saturate, infection via proteoglycan is much less efficient than infection via ICAM-1, which is present at much lower concentrations. This might point to differences in virus uptake, uncoating, and/or routing to the site most efficient for RNA release and might explain why the ubiquitously and strongly expressed HS is not a good receptor for efficient infection. Whereas uptake by ICAM-1 occurs via the clathrin-coated pit pathway (3), linkage of the glycosaminoglycans to glycosyl-phosphatidylinositol-anchored proteins might direct the virus to lipid rafts (31). Further work will be aimed at differentiating the pathways followed by this virus when taken up by ICAM-1 compared to HS.

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