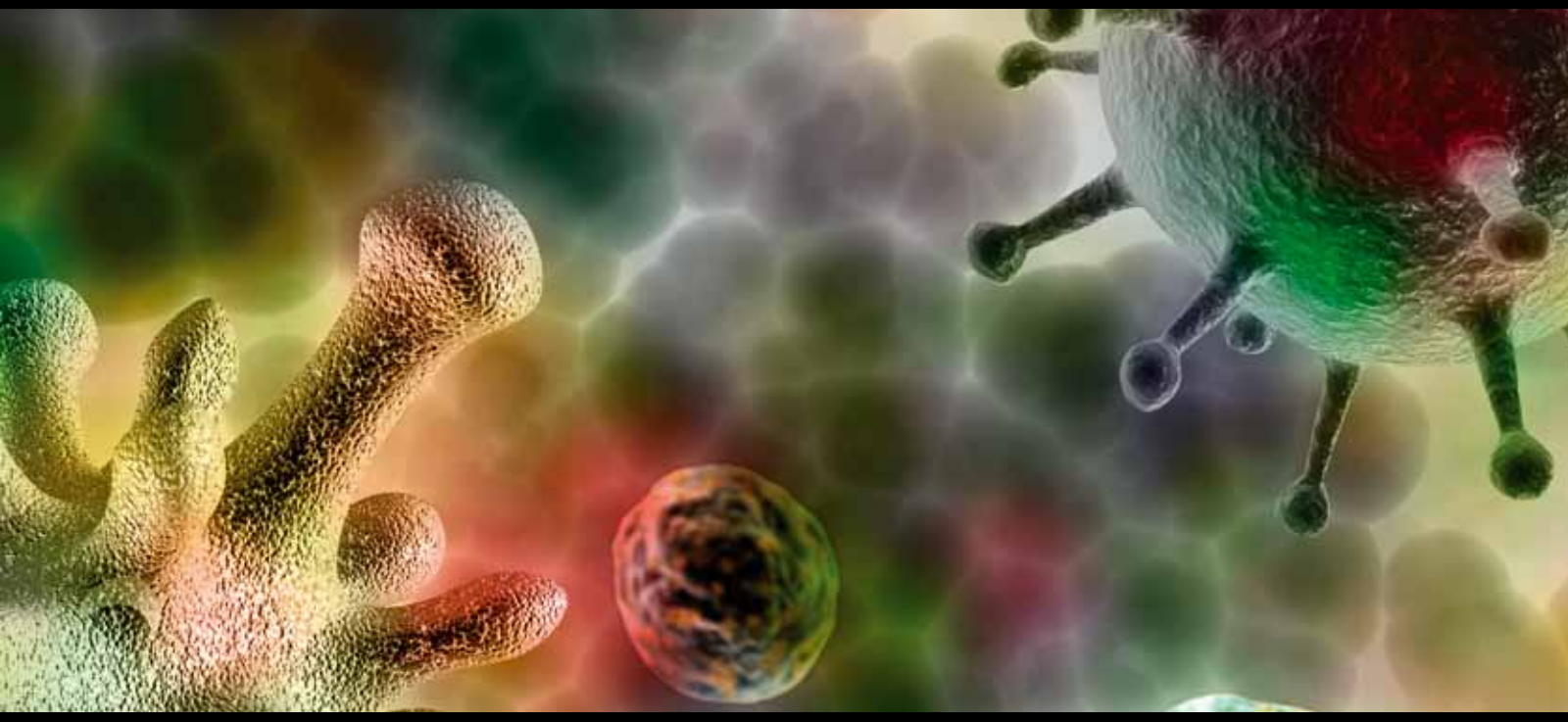
Advances in Virology

Virus Entry by Endocytosis

Guest Editors: Anthony V. Nicola, Hector C. Aguilar, Jason Mercer, Brent Ryckman, and Christopher M. Wiethoff



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Editorial Board

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Editorial

Virus Entry by Endocytosis

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Virus entry is the process by which the incoming viral particle gains access to the host cell. Investigation of viral entry reveals novel targets for interfering with the virus before it can commandeer the host cell machinery for replication. Viruses use virally encoded and cellular factors to overcome multiple obstacles for successful entry, allowing for the delivery of the viral genome to the preferred subcellular site of viral replication. Virus-receptor interactions can play multiple roles in viral entry, including cell attachment, endocytosis, membrane fusion, and molecular signaling. Endocytosis is a fundamental cellular activity that is utilized by the majority of animal virus families to introduce their genetic material to the cell interior.

The different pathways subjugated by viruses include clathrin-mediated endocytosis, caveolae, macropinocytosis, and nonclathrin, noncaveolae routes. Endocytic processes offer several advantages to the incoming virus, including transit through the cell periphery, which can contain a thick layer of cortical actin. The acidic environment of an endosomal compartment is often needed to mediate penetration into the cytoplasm. This special issue on viral entry via endocytosis compiles five topical review articles on several diverse animal viruses.

Two papers in this special issue review the entry of members of the nonenveloped picornavirus family. R. Fuchs and D. Blaas focus on the entry of types A, B, and C human rhinoviruses, a group of more than 100 distinct serotypes for which there are no vaccines. For nonenveloped viruses, delivery of genetic cargo to the cytoplasm can occur by pore formation or rupture of an endocytic membrane. Continuing on this theme, P. Merilahti et al. review the cellular entry pathways utilized by three additional human picornaviruses: coxsackievirus A9, echovirus 9, and parechovirus 1. These viruses share the common ability to bind to host cell integrins during viral entry.

Herpesviral entry is a complex interplay of multiple viral envelope proteins and multiple cellular factors. In the third paper, T. Maeki and Y. Mori present a review of the cell entry of two closely related betaherpesviruses, human herpesvirus-(HHV-)6A and HHV-6B. Interactions of both HHV-6 species with the host cell receptor CD46 are considered. These viruses contain distinct glycoprotein complexes comprised of gH-gL and additional proteins. This is a characteristic shared by other human herpesviruses, such as human cytomegalovirus and Epstein-Barr virus. HHV-6A and HHV-6B both contain complexes of gH/gL/gO and gH/gL/gQ1/gQ2.

In another paper, E. Rumschlag-Booms and L. Rong review the host cell entry of the global respiratory pathogen influenza A virus. Sialic acid moieties are critical for influenza pathogenesis, serving as binding receptors for entry and as determinants of tropism. Focus is on the genetics of influenza A viruses that contribute to entry tropism and on developing anti-influenza entry therapeutics and their mechanism

of action. Endocytosis is an important pathway for the entry of several retroviruses. In a paper of this special issue, Y. Kubo et al. review the role of endosomal acidification and cathepsin proteases in retroviral entry with an emphasis on murine leukemia viruses (MLVs) and human immunodeficiency virus (HIV). Reports of the pH-dependent entry of CD4-dependent and -independent HIVs are also discussed.

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Erratum

Erratum to "Endocytosis of Integrin-Binding Human Picornaviruses"

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Due to unfortunate errors at the proof-reading stage, there are several misplaced references. A list of correct references in specified sentences is provided here as follows.

Page 3: binding of E-1 to integrin $\alpha 2\beta 1$ does not induce uncoating but instead may lead to the stabilization of capsid suggesting that viral RNA is released during endocytosis and not on plasma membrane [54, 60].

Page 3: this was based on the virus accumulation in caveolin-1-positive endosomes in SAOS cells overexpressing integrin $\alpha 2\beta 1$ [60, 66]. However, at the same time and using another cell model, CV-1, the same authors demonstrated that majority of E-1 do not colocalize with caveolin-1 on the plasma membrane [67]. This observation was based on parallel comparisons to SV40, which is known to use caveolar route at least in some cell lines [62].

Page 4: dominant-negative caveolin-3 has been shown to block E-1 infection [68].

Page 4: which are localized in early endosomes and function in MVB formation [69].

Page 4: the recent finding that ESCRT complex recruits caveolin-1 into maturing intralumenal vesicles may explain why E-1 and caveolin-1 are found in similar structures early in infection [66, 69].

Page 5: we recently showed that CV-A9 internalization is dependent on $\beta 2$-microglobulin [72].

Page 5: Arf6 (ADP-ribosylation factor 6) is a small GTPase, which has multiple roles in the regulation of membrane traffic and other cellular functions, but it was only recently when it was linked to virus endocytosis [72].

Page 5: and this may explain why it remains highly pathogenic [75, 76].

Page 5: which is evidently in contradiction with the suggestion that HPeV-1 is endocytosed via clathrin-mediated pathway [105]. On the other hand, MHC I (with $\beta 2$M) has been linked to internalization of $\beta 1$-integrins, but previously not shown to be involved in HPeV-1 infection [105].

Page 9: the data in reference [83] should be as follows: O. Heikkilä, E. Karelehto, P. Merilahti et al., "HSPA5 protein (GRP78) and b2-microglobulin mediate internalization and entry of coxsackievirus A9 via a novel Arf6-dependent entry pathway in human epithelial colon adenocarcinoma cells," Submitted.

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Review Article

Influenza A Virus Entry: Implications in Virulence and Future Therapeutics

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Influenza A viruses have broad host tropism, being able to infect a range of hosts from wild fowl to swine to humans. This broad tropism makes highly pathogenic influenza A strains, such as H5N1, potentially dangerous to humans if they gain the ability to jump from an animal reservoir to humans. How influenza A viruses are able to jump the species barrier is incompletely understood due to the complex genetic nature of the viral surface glycoprotein, hemagglutinin, which mediates entry, combined with the virus's ability to use various receptor linkages. Current therapeutics against influenza A include those that target the uncoating process after entry as well as those that prevent viral budding. While there are therapeutics in development that target entry, currently there are none clinically available. We review here the genetics of influenza A viruses that contribute to entry tropism, how these genetic alterations may contribute to receptor usage and species tropism, as well as how novel therapeutics can be developed that target the major surface glycoprotein, hemagglutinin.

1. Introduction

Influenza viruses belong to the Orthomyxoviridae family, which consists of several genera. The first includes both influenza A and B viruses, while another is comprised of influenza C virus [1]. These classifications are based on the distinct antigenic nature of the internal nucleoprotein and matrix proteins of each virus. Infection with influenza subtypes B and C is mostly restricted to humans [2, 3], while subtype A is able to infect a wide range of hosts including but not limited to humans, swine, horses, domestic and wild birds, fowl, and dogs [4–8]. This broad spectrum of hosts plays a pivotal role in the ability of the virus to reassort, mutate, and spread, all of which contribute to the ever-present global threat of influenza.

Influenza A virus poses the most serious hazard of the three subtypes, causing global economic losses as well as severe health concerns. Influenza A virus is the causative agent of severe respiratory illness infecting nearly 15% of the world's population with upwards of 250,00–500,000 deaths estimated by the World Health Organization. Infections are characterized by upper respiratory distress along with high fever, myalgia, headache and severe malaise, nonproductive cough, sore throat, and rhinitis. Severe illness and death are mainly associated with the young, elderly, and those with compromised immune systems [2].

Influenza viruses have ravaged human and poultry populations around the world for centuries, causing serious illness and death, major economic loss, in addition to instilling fear as the next potential deadly pandemic. During the twentieth century, this virus caused three major pandemics, which resulted in an estimated 20–50 million deaths combined worldwide [9–11]. In the twenty-first century, 2009 Pandemic H1N1 was caused by a reassorted swine strain. The reassortment included influenza viruses of human, avian, and two swine strains [12]. The resultant reassorted swine strain then jumped to humans, spreading around the world within a few weeks [12, 13]. The initial result of this event was more than 22 million reported cases, 13,000 deaths, the blocking of countries' borders, and the closing of numerous

schools [14]. A recent study suggests the actual impact may be more than 10 times the initial estimates [15]. While we are currently in the postpandemic phase, this H1N1 strain is the currently circulating endemic influenza strain among human populations. Upwards of 20–40% of the world's population is thought to have immunological protection for the time being, as they have already been exposed to the virus.

Influenza viruses possess several unique characteristics, many of which potentiate the menace posed by this virus. One such feature is the segmented nature of the viral genome [16]. The virus carries eight negative-sense RNA segments. Due to the segmented nature of the viral RNA, if a host cell is infected with two viruses of different influenza strains, the gene segments of one virus can recombine with those of another virus during replication. This reassortment event is referred to as antigenic shift. The newly formed virus can be especially dangerous if a human adapted strain acquires gene(s) which transform it into a highly pathogenic strain, or if a highly pathogenic strain acquires the necessary gene(s) to infect and spread amongst humans. Either scenario is predicted to raise serious threats worldwide, as was the case in 1957 and 1968 [17, 18].

The major influenza pandemics in the twentieth century, along with the 2009 Pandemic H1N1, are thought to have arisen via antigenic shift. The pandemic of 1957, better known as "Asian Influenza" H2N2 virus, was originated in Southern China and spread rapidly to the United States and Europe causing more than 1 million deaths worldwide [19]. Sequence analysis along with biochemical studies suggest that this particular virus was originated from the reassortment or genetic mixing of an avian virus with that of a human virus [19–22]. While the recombinant virus was not particularly virulent, the high level of mortality associated with it is attributed to the immunological naivety of the infected populations. A similar scenario was seen with the pandemic of 1968, the "Hong Kong Influenza." The HA gene of this virus was of the H3 subtype and originated from an avian source along with the PB1 viral polymerase protein [21, 23–25]. These two avian gene segments reassorted with a human virus, creating a new virus with greater virulence and the ability to infect humans. Furthermore, human populations were immunologically naïve to this recombinant virus, making the health impact that much greater. Much devastation and loss are attributed to pandemics arising from antigenic shift and it is antigenic shift that is predicted to be the likely cause of the next pandemic [26]. Furthermore, evidence points to antigenic shift as the perpetrator of the most severe of the influenza pandemics. It is believed that antigenic shift was responsible for the first and most severe pandemic of the 20th century in 1918, killing an estimated 50 million people worldwide [27–32]. Recent sequence analysis of this H1N1 virus, referred to as the "Spanish Flu", strongly suggests that the virus was directly transmitted to humans from an avian source [32].

While antigenic shift is a powerful means of acquiring genetic change, antigenic drift results in more subtle changes in the genome. Antigenic drift in influenza viruses refers to residue substitutions in the virus' coding sequence via point mutations [33]. Due to the lack of a proofreading function in the RNA polymerase, influenza constantly accumulates mutations within its genome during replication. These mutations may be silent or they may alter the virulence and pathogenicity of the virus. For instance, if a highly pathogenic avian virus acquires the necessary mutations that facilitate its ability to efficiently enter and replicate in humans, then the virus can become a serious threat to humans.

The viral surface is studded with two major surface spike glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which differ greatly in genetic variation [8]. In addition, an essential ion channel protein, M2, exists on the virion surface. HA and NA exist on the virion surface in a ratio of approximately 4/5 : 1, with an estimated 400–600 total spikes. HA is responsible for mediating entry into target cells via the host cell receptor, sialic acid (SA). NA plays a major role during the budding process by releasing progeny virions from the host cell. To date, 16 subtypes of HA and 9 subtypes of NA have been identified [34, 35]. These subtypes have been mainly identified amongst different avian species, as birds are the natural reservoirs of influenza virus [7, 8]. Since entry is the first requirement for infection, it is crucial that we understand its role in host tropism, pathogenesis, as well as the role of differences between HA subtypes and species-specific viruses. Furthermore, HA has garnered recent attention as a target for broad-spectrum neutralizing antibodies [36, 37].

HA has been shown to be an important determinant for influenza virus virulence and pathogenesis. Genomic studies of the 1957 (H2N2) and 1968 (H3N2) pandemics revealed that a major contribution to virulence was due to the exchange of the HA segments between human and avian strains [24]. Sequence comparison of the 1918 (H1N1) virus to other influenza A viruses from various species reveals that the entire 1918 virus is more closely related to avian influenza A viruses than with any other species, namely humans, suggesting that accumulated mutations in the avian HA gene allowed it to better adapt to the human host. The critical role of mutations within the avian virus genome underlies the importance of studying mutations within the H5N1 virus genome that may be critical to sustain infection in and among humans although no sustained human-to-human transmission has been reported yet [38, 39].

HA exists on the virion surface as a trimer of HA_1 and HA_2 subunits linked by disulfide bonding. This surface glycoprotein is first synthesized as a single polypeptide (HA_0) of approximately 550 amino acids, which is highly N-glycosylated. HA assembles into a trimer in the rough endoplasmic reticulum (ER) before passing through the Golgi complex on its way to the cell membrane. For the virus to be infectious, the HA_0 precursor protein must be cleaved into its subunits, HA_1 and HA_2 [40, 41]. If HA is not cleaved, fusion of the viral envelope with the endosomal membrane cannot occur, thus the genomic contents cannot be released within the target cell. At the structural level, cleavage of HA_0 is important because it reveals the hydrophobic N-terminus of HA_2, the fusion peptide, which is inserted into the host membrane during HA-mediated viral/host membrane fusion and viral entry. Upon endocytosis, the acidic pH (5–5.5)

environment triggers HA_2 to undergo the irreversible [42–45] conformational changes necessary for fusion to occur, allowing the viral and host membranes to fuse, releasing the viral genomic contents into the cytoplasm to begin replication [46].

Two known classes of proteases are involved in HA_0 cleavage [47–49]. The first known protease class recognizes a single arginine at the cleavage site. HA with such a cleavage site is processed at the cellular surface during the budding process or on released viral particles by secretory proteases, such as tryptase Clara, a trypsin-like protease found in the alveolar fluid of rat lungs, plasmin, and bacterial proteases [50]. This particular set of trypsin-like enzymes is found either in specialized cells or within specific organs, thus viruses carrying such an HA have more restricted activation, infection capability, and therefore limited replication and spread [40, 41, 50]. Due to this restriction, trypsin-like activated viruses are generally thought to be less pathogenic. It is interesting to note that while low pathogenicity is generally associated with a virus whose HA has the trypsin-like cleavage site, the most highly pathogenic human influenza virus was restricted to trypsin-like enzyme cleavage [51]. The enzyme-limited, restricted sites of replication correspond with sites of natural infection for humans and birds, that being limited to the upper-respiratory tract (humans) or gastrointestinal tracts (birds) [20].

The other variant of HA contains a polybasic consensus sequence cleavage site, R-X-K/R-R, which is recognized by the subtilisin-like endoproteases, furin, and PC5/6 [52, 53]. This protease is expressed in the trans-Golgi network, therefore the HA is activated during the exocytic route during virus maturation [53, 54]. As this protease is nearly ubiquitously expressed, a virus with this cleavage site can replicate throughout a host and cause extensive systemic spread. Due to this characteristic, viruses with the polybasic site are generally considered highly pathogenic strains, such as the H5 and H7 strains [53, 55]. A comparative analysis at the entry level between a low pathogenic H5N2 strain and a highly pathogenic H5N1 strain revealed that the major restriction to entry was the cleave site sequence. Replacement of the monobasic cleavage site into the polybasic cleavage site on HA enhanced the HA-mediated entry [56]. However, the worst human influenza epidemic recorded, the 1918 Spanish Flu, did not have the polybasic cleavage site as previously discussed. Instead, it had a single arginine, highlighting the complicated nature of influenza viruses and their virulence and pathogenicity [51].

In addition to the aforementioned two classes of proteases, the following proteolytic enzymes are implicated in HA activation: type II transmembrane serine proteases (TTSPs) TMPRSS2, TMPRSS4, and human airway trypsin-like protease (HAT) [47]. While less is known about these proteases, their activity may be additionally associated with viral pathogenicity and tropism.

Influenza entry tropism is mainly determined by the binding preference of HA_1 to its receptor, SA. SA has long been believed to be the sole receptor for the influenza virus. It was discovered nearly 70 years ago that upon addition of influenza virus to chicken erythrocytes, the cells would agglutinate at 4°C [57]. A shift in temperature to 37°C would cause the virus to elute, while addition of new influenza virus no longer caused agglutination. This phenomenon suggested that, in addition to binding a surface molecule on the erythrocytes, the virus carries a receptor-destroying enzyme. It was later discovered that the cellular component removed by the virus was SA and that treatment of erythrocytes with purified sialidase from *Vibrio cholerae* prevented agglutination [58–60]. This finding was the first demonstration that SA acts as a receptor for influenza A viruses.

SA encompasses a large family of sugar molecules. The most prevalent member of this family is N-acetylneuraminic acid (NeuAc). It primarily exists as a six-carbon ring with several unique components extending from the ring. The most important feature of SA, with regards to influenza, is the manner in which the free sugar is attached to the host cell surface. Host cells carry various surface glycoproteins and glycolipids, many of which are highly modified. These surface proteins that are modified with a terminal SA play a crucial role in influenza entry, serving as the viral attachment and entry receptor. SA can be attached to the underlying glycocalyx in one of three main linkage patterns, either α2,3, α2,6, or α2,8 [61]. While other linkages exist, these three are the most prevalent in mammalian cells [62].

In addition to viral entry, SA plays an equally important role in determining host tropism. Influenza tropism is highly influenced by the linkage of SA, with avian and human viruses preferentially utilizing different linkages. Avian viruses have been classified as predominantly α2,3 specific, while human viruses tend to favor the α2,6 linkage [63–66]. These preferences have been established from studies examining SA distribution within a specific host, binding of the virus to SA at the protein level, as well as studies analyzing replication efficiency in the presence of these specific linkages. Increased prevalence of the α2,3-linked SA in the avian gastrointestinal tract correlates with the ability of the virus to enter and replicate here, as it is the site of natural infection for birds [23, 61]. As the natural carrier of the virus, avian populations usually display few disease symptoms [7]. Due to the high prevalence of receptors within the gastrointestinal tract, the virus replicates most efficiently there and is excreted through waste products. On the other hand, human influenza viruses predominantly utilize SA in α2,6 conformation which is highly prevalent in the upper respiratory tract [23, 65, 67, 68]. SA linkages within the human respiratory tract are present on nasal mucosal epithelial cells, paranasal sinuses, pharynx, trachea, and bronchi, all carrying α2,6 SA while α2,3 SA is found on nonciliated cuboidal bronchi and alveolus, as well as type II cells within the alveolar wall [69]. Based on these data, it is the low levels of α2,3 linkages in the upper tract that have been postulated as being a block for efficient infection and replication of α2,3 preferring viruses in humans. On the other hand, flow cytometry studies using lectins specific for α2,3 and α2,6 SA showed that both linkages were present on human bronchial epithelial cells with α2,6 SA being the vast majority [70, 71], suggesting another layer in the complexity of influenza tropism beyond SA preference. In addition, it still remains unclear if all subtypes of influenza virus target the same subset of respiratory cells and/or have the same affinity

and avidity for α2,3 and α2,6 SA linkages. Besides terminal sialic-acid linkages, specificity is also influenced by internal linkages along with modification of inner oligosaccharides including sulfation, fucosylation, and sialylation [72, 73]. Overcoming this binding restriction may be one step needed for avian influenza to more efficiently spread from human to human.

The linkage of SA and its influence on influenza entry has been extensively characterized to determine its role in tropism; however in early 2008 an additional level of complexity was revealed. Chandrasekaran et al. reported that the crucial determinant for influenza tropism is the structure of the underlying glycocalyx, not the terminal SA linkage [74]. Using a series of analyses, it was reported that avian viruses prefer SA in a cone-like topology. This shape is adopted by SAs, both α2,3 and α2,6, with short underlying glycan(s) and allows HA to contact Neu5Ac and galactose sugars in a trisaccharide motif. Human viruses are reported to prefer an umbrella-like glycan topology, which is unique to α2,6 SAs with long underlying glycans. This report also concluded that α2,6 alone is insufficient for human transmission, as avian viruses can utilize α2,6 SA on a short sugar chain, suggesting that the virus must adopt the ability to utilize α2,6 SA on a long sugar chain. It was concluded that it is the glycan composition, and thus the SA topology that may influence influenza tropism, not just the SA linkage present.

The region of HA that is responsible for binding SA is referred to as the receptor-binding domain (RBD). This pocket-shaped depression is located at the membrane-distal tip of each monomer within the trimeric HA structure and is comprised of the 190 helix (residues 190–198), the 130 loop (residues 135–138), and the 220 loop (residues 221–228) based on H3 numbering [25]. Several key conserved residues including tyrosine 98, tryptophan 153, and histidine 183 form the base of the binding pocket and are crucial for maintaining the structural basis of the binding pocket as well as in forming interactions with SA [75]. Sequence analysis from several strains of influenza along with structural modeling has given great insight into the residues that play a pivotal role in SA binding preference [25, 76].

Structural studies of HA suggest that avian and human influenza viruses appear to be distinct in their RBDs at positions 226 and 228 [77, 78]. Avian HAs tend to have glutamine and glycine at the respective positions while human HAs carry leucine and serine [78–81]. The avian HA with these residues forms a narrow binding pocket for the α2,3 receptor while the change in residues for human HAs results in a broader pocket for the α2,6 receptor. Neither position 226 nor 228 plays a direct role in binding, rather they seem to influence the contour of the pocket [70, 82]. These differences in pocket shape correlate well with the glycan topology studies. Additionally, it was shown that a lab-adapted strain which prefers α2,6 binding was able to switch preferences to α2,3 when grown in the presence of α-2 macroglobulin, a α2,6 glycoprotein found in high concentrations in horse sera [65]. Based on these and other studies, it seems that residue 226 and 228 have an important indirect role in SA binding compatibility and therefore preference.

Interestingly, the 1918 H1N1 virus HA carried glutamine 226 and glycine 228 corresponding to the avian α2,3 receptor, however the virus is able to bind the α2,6 receptor, demonstrating that changes at these positions are not necessary for altered receptor binding [30]. Further analysis of the 1918 HA revealed that a single change from asparagine to glutamate at position 190 was responsible for the altered binding phenotype [30]. In addition, a change from asparagine to glycine at 225 in combination with the change at 190 increased respiratory transmission of the virus in the ferret model, further highlighting the importance of the RBD residues.

More extensive studies focusing on the H3 subtype revealed that human viruses with α2,6 preference have leucine at 226 and serine at 228, while avian viruses with α2,3 preference have glutamine at 226 and glycine at 228 [63, 82]. Residues 193 and 218 have also been implicated as important determinants for receptor preference in the H3 subtype, however specific residue changes have not been fully established [83].

Similarly, studies focusing on seasonally circulating H1 viruses found avian and human viruses differ at two positions in their binding preference. A proline at 186 and a glycine at 225 correspond with α2,3 type binding, while serine 186 and asparagine 225 are favored by α2,6 type binding [25]. Interestingly, HA proteins of both avian and human viruses have glutamine at 226 and glycine at 228. The discrepancies highlighted by the H3 and H1 studies in combination with the studies on the H1N1 Spanish Flu illustrate the complex nature of receptor binding, that is, not all influenza A viruses behave in a similar way, not even among avian strains nor human strains.

Since the initial H5N1 influenza outbreak that began in China in 1997, several studies have focused on the RBD of this particular viral strain. These studies include the use of sialoglycoconjugates, crystal HA structures, and simulated computer-based binding assays [55, 69, 72, 74, 84–87]. Residues that have been implicated in human receptor-type binding include asparagine 227, asparagine 159, lysine 182, and arginine 192. It is proposed that residues 159, 182, and 192 influence binding by stabilizing the HA binding pocket and maintaining structural integrity, as these residues are not in direct contact with SA. A switch of glutamine to leucine at position 226 and a switch of glycine to serine at position 228 equates a shift from avian receptor to human receptor specificity, as seen in the H3 virus as well [86, 88]. Further studies specifically targeting the HA of the A/Vietnam/1203/2004 H5N1 virus demonstrate the importance of mutation E190D which reduced the binding to 2,3 linkages, as well as the double mutant Q226L/G228S [86]. In addition the H5N1 HA surface residue tyrosine 161 has recently been implicated in altering glycoconjugate recognition and cell-type dependent entry. Substitution of tyrosine 161 to alanine switched the binding preference from N-acetylneuraminic acid to N-glycolylneuraminic acid [87]. It is important to note that different strains of the H5N1 virus from different time points during the virus outbreak were used in these studies.

To better understand the role of naturally acquired mutations and their ability to potentiate sustained transmission, two recent studies showed that as few as four to five amino

acid substitutions along with gene reassortment may be sufficient for respiratory droplet transmission between ferrets [89, 90]. Herfst et al. identified (based on H5 numbering) glutamine to leucine at 222 and glycine to serine at 224 as critical residues to alter sialic acid specificity from the avian α2,3 SA to the human α2,6 SA. Two additional HA substitutions, threonine to alanine at 156 and histidine to tyrosine at 103 play a role in disrupting N-linked glycosylation and monomer interaction, respectively. Lastly, a switch from glutamate to lysine at 627 in the polymerase PB2 subunit was identified, which is a common change seen in mammalian adapted influenza strains. Imai et al. identified four substitutions, all within HA. Similar to the results of Herfst et al., it was found that substitutions of asparagine to lysine at 220 and glutamine to leucine at 222 can alter SA specificity from the avian α2,3 SA to the human α2,6 SA. Again, a disruption in N-linked glycosylation via asparagine to aspartate at position 154 was identified along with a change in the stalk region corresponding to threonine to isoleucine at 315.

While the linkage of SA and the residues within the RBD plays a significant role in viral entry, the glycoconjugate to which SA is attached appears to be an additional important factor. Chu and Whittaker determined that cells deficient in the GnT1 gene lack terminal N-linked glycosylation, rendering them deficient in influenza A viral entry [91]. The GnT1 gene encodes the enzyme N-acetylglucosaminyltransferase, which is involved in modification of N-linked glycans in the Golgi apparatus. These cells lack N-glycoproteins, but still possess surface α2,3 SA and α2,6 SA. While considering this phenotype, this mutant Chinese Hamster Ovary (CHO) cell line has the capacity to bind HA and expresses sufficient levels of both SA linkages on the cell surface for attachment. In this mutant CHO cell line, complete entry was blocked, as virions were not endocytosed. These results suggest a specific role for N-linked glycoproteins in influenza A entry, perhaps acting as a cofactor in mediating entry.

There is evidence suggesting that SA is not necessary for infection by influenza. The HA of a human H1N1 strain was shown to bind glycoconjugates other than SA [92]. In addition, Stray et al. demonstrated the ability of desialylated Madin Darby canine kidney (MDCK) cells to be infected by influenza A virus [93]. A study by Nicholls et al. demonstrated the ability of the H5N1 virus to infect upper and lower respiratory tract cells in the presence or absence of α2,3 SA which is believed to be the entry receptor for this virus [94]. In addition, the levels of SA on susceptible and nonsusceptible cells do not correlate with either α2,3 SA or α2,6 SA for an H5N1 strain [95]. Taken together, it is possible that the barrier to efficient human infection and human-to-human transmission of the H5N1 virus is not due to SA linkages, but rather it is due to inefficient use or expression of a yet unidentified entry mediator.

To fight the spread of influenza, prophylactic therapeutics and vaccines continue to be vital methods of control. Vaccines are the primary means of controlling the spread of the virus and are based on inactivated viruses, live attenuated viruses, or purified viral protein(s) that illicit a strong neutralizing antibody response [96]. Of these vaccines, most target the globular head region of HA, containing the receptor-binding domain, thus preventing attachment of the virus to susceptible cells. These neutralizing antibodies are rarely immunoresponsive to an alternate influenza strain, often losing their potency as their corresponding strain acquires mutations during circulation. Furthermore, due to the virus ability to constantly acquire genetic changes, it is difficult to predict what the circulating strain(s) for the upcoming year will be. A mismatch of vaccine strain with circulating strain(s) will offer little to no protection.

In addition to vaccines, anti-flu therapeutics have been developed which can be divided into two classes, anti-NA and anti-M2 [97]. The first class of inhibitors specifically targets the NA protein of the virus, halting the spread of progeny virions [98]. During the budding process of influenza, newly produced progeny virions are tethered to the host cell surface via HA proteins interacting with SA molecules. NA functions in recognizing this HA-SA interaction and cleaves the SA moiety, releasing the viral particle [99]. The currently approved therapeutics for influenza infection include NA inhibitors which block this step, thus preventing release and further spread of the virus, both within the infected host and consequently to others. Included in this category of antivirals are two of the most commonly used therapies, Zanamivir (trade name Relenza) and oseltamivir phosphate (trade name Tamiflu) for the treatment and prevention of influenza A and B viruses. Zanamivir and oseltamivir phosphate are competitive inhibitors for the active site of the NA enzyme [100]. While Zanamivir and oseltamivir phosphate can be highly effective in both treating influenza and in outbreak control, in the 2008-2009 flu season, nearly 100% of H1N1 samples tested by the Center for Disease Control (CDC) were shown to be resistant to oseltamivir phosphate [101, 102]. This high level of resistance highlights the need to develop new antivirals against influenza.

Another class of influenza inhibitors, the adamantanes, is those which block the M2 ion channel on the virion surface. The M2 ion channel is embedded within the virion lipid bilayer and facilitates hydrogen ion transport, ultimately leading to virion uncoating and replication during the entry process [103]. The adamantane derivatives, amantadine and rimantadine, were approved for the treatment and prevention of influenza A only, as only influenza A class viruses have the M2 protein [103]. The CDC and WHO report that greater than 99% of circulating strains are resistant to M2 inhibitors and have recommended their use to be discontinued. Therefore these inhibitors are only used when a specific nonresistant strain is thought to be the causative infectious agent [101]. These antivirals, along with the NA inhibitors, provide a treatment option after infection, however since not all influenza A viruses respond to these treatments, these drugs may be ineffective. In addition, resistance to these treatments over the course of an outbreak or influenza season underlies the urgency to develop new antiviral therapies.

Since there is a lack of clinically available inhibitor(s) which targets the major viral surface protein, HA, recent clinical trials are pushing forward to expand the repertoire of

influenza therapeutics, including drugs that target HA, which is the target of most neutralizing antibodies [104]. These new drugs include cyanovirin-N and thiazolides. Cyanovirin-N targets the high-mannose oligosaccharides, neutralizing the viral particle [104, 105]. The thiazolides work to block the maturation of HA posttranslationally [104, 106]. Recently, several groups have identified broad-spectrum neutralizing antibodies that are protective against group 1 (which includes HA subtypes 1, 2, 5, 6, 8, 9, 11, 12, 13, and 16) and group 2 (which includes HA subtypes 3, 4, 7, 10, 14, and 15) influenza A viruses [36, 107–109]. These broad-spectrum neutralizing antibodies (referred to as CR6261, F10, and F16) are further distinct in that they target the stem region of the HA molecule. The proposed model is that by targeting the stem region, more specifically the fusion peptide, these antibodies are able to prevent exposure of the fusion peptide under acid conditions. This trapping mechanism prevents the viral membrane from fusing with the host endosomal membrane, locking the genetic contents within the viral particle. An additional stem-directed neutralizing antibody, CR8020, is active against group 2 influenza A viruses. While not as broad in activity as the aforementioned antibodies, CR8020 could be utilized in conjunction with a group 1 neutralizing antibody, providing a one-two punch against both HA subunits.

Influenza viruses will continue to circulate among animal and human populations, acquiring and exchanging genetic components as they do. Due to the constant change in their genetic profiles, influenza viruses will continue to pose a serious threat, from both an economic and public health point of view. Continued study and surveillance of influenza viruses is further highlighted by our inability to maintain effective influenza vaccines and prophylactic therapeutics. The prospective of new antivirals, especially those that provide broad-spectrum protection, provide renewed efforts in our ability to control influenza infection and spread.

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Review Article

Retrovirus Entry by Endocytosis and Cathepsin Proteases

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Retroviruses include infectious agents inducing severe diseases in humans and animals. In addition, retroviruses are widely used as tools to transfer genes of interest to target cells. Understanding the entry mechanism of retroviruses contributes to developments of novel therapeutic approaches against retrovirus-induced diseases and efficient exploitation of retroviral vectors. Entry of enveloped viruses into host cell cytoplasm is achieved by fusion between the viral envelope and host cell membranes at either the cell surface or intracellular vesicles. Many animal retroviruses enter host cells through endosomes and require endosome acidification. Ecotropic murine leukemia virus entry requires cathepsin proteases activated by the endosome acidification. CD4-dependent human immunodeficiency virus (HIV) infection is thought to occur via endosomes, but endosome acidification is not necessary for the entry whereas entry of CD4-independent HIVs, which are thought to be prototypes of CD4-dependent viruses, is low pH dependent. There are several controversial results on the retroviral entry pathways. Because endocytosis and endosome acidification are complicatedly controlled by cellular mechanisms, the retrovirus entry pathways may be different in different cell lines.

1. Introduction

Retroviruses include many pathogenic agents in humans and animals. Human immunodeficiency virus (HIV) and human T-cell leukemia virus (HTLV) induce acquired immunodeficiency syndrome (AIDS) and adult T-cell leukemia (ATL), respectively. Murine leukemia viruses (MLVs) are also well-studied among retroviruses because the MLVs are used comparatively as animal models of several human diseases (leukemia, immunodeficiency, and neuropathogenic diseases) and as gene transfer tools. In addition, there are animal retroviruses that are important problems in the livestock industry, such as Visna, equine infectious anemia virus, bovine leukemia virus, and Jaagsiekte sheep retrovirus.

Retroviruses contain envelope membranes consisting of lipid bilayers derived from virus-producing cells. Genomes of simple retroviruses such as MLVs encode three essential elements, gag, pol, and env genes. Complex retroviruses including HIV additionally encode accessory genes whose products regulate the retroviral expression and suppress host antivirus factors [1]. The gag and pol genes encode viral structural proteins and enzymes, respectively. These proteins are synthesized as precursor polyproteins and then are cleaved to mature peptides by a protease encoded by the retroviral pol gene.

Retroviral envelope (Env) glycoprotein encoded by the env gene is also synthesized as a precursor protein and is cleaved to surface (SU) and transmembrane (TM) subunits by a cellular protease [2]. Retroviruses enter host cells by fusion between viral envelope and host cell membrane, following the recognition of cognate cell surface receptors. The SU protein binds to the cell surface receptor protein. The TM protein anchors the SU protein to the surface of viral particles and virus-producing cells by the complex formation of SU and TM. The TM protein mediates the membrane fusion reaction. The entry mechanisms of retroviruses are vigorously studied but are not completely understood. Elucidation of the retrovirus entry machinery

would contribute to the development of new therapeutic approaches for retrovirus-induced diseases.

2. Membrane Fusion by Retroviral Env Glycoprotein

Mechanism of membrane fusion by the retroviral TM proteins is described elsewhere in details [3–7] and is similar to those used by envelope proteins of other enveloped viruses [8, 9]. Briefly, the retroviral entry mechanism is proposed as follows. The TM protein is thought to have hairpin-like structure (Figure 1). The binding of SU with its cognate cell surface receptor induces conformational changes of the TM subunit. The N-terminal hydrophobic domain of the TM subunit called fusion peptide is exposed by the conformational change and inserted into host cell membrane. The TM protein then coverts to a trimer-of-hairpins conformation, and viral envelope and host cell membranes approach and mix. Finally, the fusion pore is formed and expanded to derive the viral core into host cell cytoplasm. This conformational change pathway of the TM protein induces the membrane fusion for the retroviral entry into host cells.

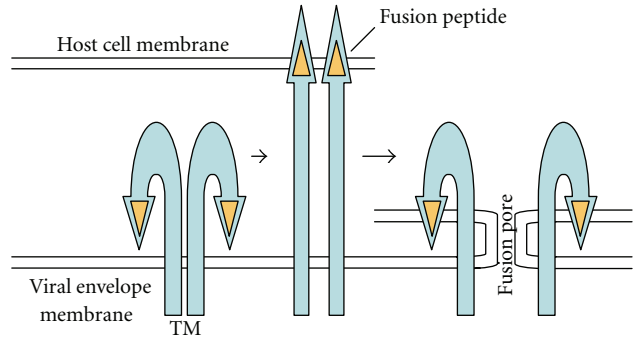

Figure 1: Conformational change of retroviral TM subunit for membrane fusion.

3. Retrovirus Receptors

In this section, we will mainly focus on the infection receptors for MLV and HIV, with which entry mechanisms are most extensively studied among retroviruses. Other reviews should be referred to concerning the infection receptors of animal retroviruses in general [10, 11]. MLVs are divided into four groups according to their host ranges and infection interference, and the four groups recognize different cell surface receptors. Ecotropic MLVs infect mouse and rat and bind to cationic amino acid transporter 1 (CAT1) as the infection receptor [12]. Amphotropic MLVs infect many types of mammals, and inorganic phosphate symporter 2 (Pit2) is the amphotropic infection receptor [13, 14]. Polytropic MLVs has a similar host range to the amphotropic MLVs. The amphotropic MLVs cannot infect amphotropic virus-infected cells, because Pit2 are already occupied by the amphotropic Env proteins, called infection interference. Whereas the polytropic MLVs can infect amphotropic virus-infected cells, indicating that the polytropic virus receptor is different from the amphotropic receptor. Polytropic MLVs recognize XPR1 for the infection [15–17], whose physiological function is unknown yet. Xenotropic MLVs recognize the XPR1 as polytropic MLVs, but do not infect mouse cells. These MLV infection receptors are all multimembrane spanning proteins.

The infection receptors of HIV are CD4 and one of chemokine receptors (CXCR4 or CCR5) [18]. However, HIV variants that do not require CD4 for the infection are sometimes isolated from AIDS patients [19, 20] though the infectivity of CD4-independent variants is much lower than that of CD4-dependent viruses [21]. Such CD4-independent HIV variants recognize multimembrane spanning CXCR4 or CCR5 as the sole infection receptor, like the MLVs. CD4 is a single-membrane spanning protein, and HIV variants recognizing CD4 as the sole infection receptor have not been isolated. CD4-independent variants of simian immunodeficiency virus (SIV) are more frequently isolated than CD4-independent HIV variants [22, 23]. It is thought that CD4-independent HIV variants are prototypes of CD4-dependent HIVs [22–24].

4. C-Terminal Tail of Retroviral Env Protein Inhibits Membrane Fusion

When retrovirus-producing and -susceptible cells are mixed, viral Env proteins on the cells can effectively interact with infection receptors on the neighboring susceptible cells via direct cell-to-cell contact. The interactions can have both positive and negative effects on the retrovirus replication. First, they can lead to cell-to-cell infection that allows very rapid and synchronized replication of virus compared to the cell-free infection [25, 26]. This can be advantageous for the virus replication in the presence of antiviral agents [27]. Second, the interactions can induce a negative effect, that is, the rapid apoptotic cell death, via syncytium formation [28–30]. This can be disadvantageous for the virus in that the sustained production of progeny virions becomes impossible. If the apoptotic cell death proceeded more efficiently than the virus replication, it eventually would result in poor progeny virus production. Therefore, it is conceivable that the retroviruses have some mechanisms to attenuate fusion capability of the envelope TM proteins in virus-producing cells and to primarily activate it in retroviral particles upon virion budding. Consistently, such mechanisms have been suggested for the Env TM proteins of MLV and HIV.

In the case of MLV Env protein, C-terminal 16-amino acid peptide of the TM subunit called R peptide is further cleaved by the retroviral protease after the budding [31, 32]. The R peptide-containing Env protein is expressed in the virus-producing cells. The R peptide-truncated MLV Env protein can induce syncytia in susceptible cells, but the R peptide-containing Env protein cannot, indicating that the R peptide negatively regulates the syncytium formation of virus-producing cells [33, 34]. Viral particles carrying the R peptide-containing Env protein have much lower infectivity

Table 1: Inhibitors used in studies of retroviral entry pathway.

Inhibitors	Target
Ammonium chloride	Acidification of intracellular vesicles
Bafilomycin A-l	Acidification of intracellular vesicles
Concanamycin A	Acidification of intracellular vesicles
Dynasore	Dynamin-dependent endocytosis
Chlorpromazine	Clathrin-dependent endocytosis
CA-074Me	Cathepsin B protease
Dynamin DN mutant[1]	Dynamin-dependent endocytosis
Caveolin DN mutant	Caveolin-dependent endocytosis
Clathrin DN mutant	Clathrin-dependent endocytosis
Eps 15 DN mutant	Endocytosis

[1] DN: dominant negative.

than those with the R peptide-cleaved Env, showing that the R peptide cleavage during virion maturation is required for the infectivity [35–37]. It has been reported that the R peptide controls the three-dimensional structure of the SU protein [38] and a disulfide bond between the SU and TM proteins [39], suggesting that the R peptide of TM subunit regulates the receptor-mediated SU conformational changes through the S–S bond between the SU and TM. It has been recently shown that the R peptide-cleaved TM forms separated Env legs, but the R peptide ties the TM legs together [40].

Although the C-terminal domain of the HIV TM protein is not cleaved, it is suggested that interaction between the HIV TM C-terminal region and Gag precursor protein suppresses the membrane fusion activity in virus-producing cells [41]. Processing of the HIV Gag precursor after budding abrogates the suppression of membrane fusion, and the mature virions gain sufficient fusion activity for the entry. The functions of C-terminal tails of retroviral Env proteins to inhibit membrane fusion are conserved among many retroviruses [42–45], though the mechanisms are different. The C-terminal domains of retroviral Env glycoproteins function to maintain the production of progeny virions by suppressing syncytium formation-directed apoptosis of virus-producing cells.

5. PH-Dependent Retrovirus Infection

Ammonium chloride, a weak base, neutralizes acid conditions in intracellular vesicles (Table 1). Concanamycin A and bafilomycin A-1 are specific inhibitors of the ATP-dependent proton pump/vacuolar ATPase (V-ATPase) that serves to acidify endocytic vesicles [46, 47]. To analyze the pH dependence of retrovirus entry, these compounds are frequently used. Additionally these inhibitors may affect trafficking of the intracellular vesicles, because siRNA-mediated knockdowns of subunits of V-ATPase complex affect trafficking of intracellular vesicles [48]. Previously it had been reported that ammonium chloride inhibits ecotropic MLV infection but does not amphotropic and xenotropic MLV infections, showing that ecotropic MLV infection occurs through acidic vesicles, but amphotropic and xenotropic MLV infections do not [49, 50] (Table 2). The more specific inhibitors of endosome acidification (concanamycin A and bafilomycin A-1) suppress all of ecotropic, amphotropic, polytropic, and xenotropic MLV infections [51, 52]. At present, it is generally accepted that ecotropic MLV infection requires acidification, because all the studies consistently reported the suppression of ecotropic virus replication with the inhibitors of endosome acidification. In contrast, it has been shown that xenotropic MLV infections are not suppressed by bafilomycin A-1 [53] (Table 2). Due to the controversial results, the entry pathway of xenotropic MLV is not clear yet. Because different cell lines were used in those reports, the low pH requirement of the xenotropic MLV infection may be dependent on the used cell lines (see below).

In case of avian leukosis virus (ALV) infection, there are also several controversial reports. The earlier reports show that ammonium chloride and bafilomycin do not affect ALV infection, suggesting that ALV infection does not require the acidification [54, 55]. In contrast, it has been recently reported that lowering the pH results in quick and extensive cell-cell fusion by ALV [56] and that the acidification inhibitors suppress ALV infection [57, 58]. It is now thought that receptor binding of ALV induces the Env protein to convert to its prehairpin intermediate at neutral pH [59, 60], and then endosome acidification triggers the formation of the final fusion-active form of the Env protein [61–63]. It has been proposed that the discrepancy came from unusual stability of the Env prehairpin intermediate, consequent ability of fusion to proceed upon washout of the acidification inhibitors after several hours, and the relatively high pH requirement for the outer leaflet mixing [64]. Finally, it is considered that ALV entry requires endosome acidification.

The acidification inhibitors suppress infections by mouse mammary tumor virus (MMTV) [65], foamy virus [66], equine infectious anemia virus (EIAV) [67, 68], Jaagsiekte sheep retrovirus (JSRV) [69], and enzootic nasal tumor virus [70]. These results suggest that infections by many animal retroviruses are low pH dependent.

6. Internalization Pathways

The requirement of low pH for the retrovirus infections reveals that retrovirus particles are internalized into acidic intracellular compartments during virus replication. There are several different pathways for the internalization of molecules; (i) phagocytosis, (ii) macropinocytosis, (iii) clathrin- and dynamin-dependent endocytosis, (iv) caveolin- and dynamin-dependent endocytosis, (v) lipid raft- and dynamin-dependent endocytosis, (vi) clathrin-, caveolin-, and dynamin-independent endocytosis that requires lipid raft, and (vii) dynamin-, clathrin-, caveolin-, and lipid raft-independent endocytosis [48, 71]. Here we will briefly summarize the accepted mechanisms and roles of internalization, relevant to the present review [48, 72, 73].

6.1. Phagocytosis. Specialized cells such as macrophages, neutrophils, and monocytes clear debris and pathogens

Table 2: Differential dependence of HIV and MLV infections on endosome acidification.

Viruses	Dependence of acidification	Cell lines	Reference
Ecotropic MLV	Independent	Rat XC	[49, 52]
	Dependent	Mouse NIH3T3, human TE671	[49, 51, 52]
Amphotropic MLV	Independent	Mouse NIH3T3, rat XC	[49, 52]
	Dependent	Mouse NIH3T3, human TE671	[51, 52]
Polytropic MLV	Independent	Rat XC	[52]
	Dependent	Mouse NIH3T3, human RE671, rat XC	[52]
Xenotropic MLV	Independent	Human HT1080, HTX, porcine, rat XC	[49, 50, 52, 53]
	Dependent	Mouse NIH3T3, human RE671	[52]
CD4-dependent HIV	Independent	Human CEM, HeLa, C8166, VB	[49, 89–93]
	Independent	Human 293T, HeLa, TE671	[21]
CD4-independent HIV	Dependent	Human 293T, HeLa, TE671	[21]

by phagocytosis. Signaling cascades induce the actin rearrangement and form membrane extensions that cover the target particles and engulf it. Phagosomes become acidic by fusion with lysosomes (pH 5.0-6.0). Debris internalized by phagocytosis is degraded in the acidic phagosomes (phagolysosomes).

6.2. Macropinocytosis. Stimulation by certain growth factors or other signals causes membrane protrusions that fuse with the plasma membrane to form large intracellular vesicles known as macropinosomes that encapsulate large volumes of the extracellular fluid. Macropinosomes can either fuse with lysosomes (pH 5.0-6.0) or recycle back to the cell surface. There is no consensus as to the final fate of macropinosomes. Trafficking of macropinosomes seems to depend on cell type and mode of macropinocytosis induction.

6.3. Clathrin-Mediated Endocytosis. After ligands bind to their receptors, the receptor proteins are internalized into intracellular vesicles called endosomes. The endosome formation requires dynamin GTPase, and the endosomes are coated by clathrin proteins. Many receptors are segregated from their ligands in early endosomes due to weakly acidic condition (pH 6.0). Early endosomes become more acidic by V-ATPase-mediated acidification (late endosomes/lysosomes) (pH 5.0-6.0), and separated ligands are degraded by endosome proteases. Certain receptors are transferred from early endosomes to recycling endosomes (pH 6.4) and are reused on the plasma membrane. Some proteins are also recycled from late endosomes/lysosomes through the trans-Golgi network. Lysosomes often form multivescular bodies.

6.4. Caveolin-Mediated Endocytosis. Glycosylphosphatidylinositol (GPI)-anchored proteins, simian virus 40 (SV40), and cholera toxin trigger the formation of caveolae coated by caveolin proteins. These ligands are internalized into intracellular vesicles (pH 7.0) dependently on dynamin GTPase. The vesicles can be sorted to endosomes and become acidic.

6.5. Clathrin- and Caveolin-Independent Endocytosis. Cholera toxin and SV40 can also be internalized via raft microdomains into GPI-anchored protein-enriched endosomes. Mechanisms regulating this internalization pathway are unclear as of yet.

7. Internalization of Retroviral Particles into Intracellular Vesicles

A dominant negative mutant of caveolin [74], siRNA-mediated knockdown of dynamin, and a dynamin inhibitor (dynasore) (Table 1) [52] suppress the amphotropic MLV infection, suggesting that amphotropic MLV particles are internalized by the dynamin- and caveolin-dependent endocytosis for productive infection (the fourth pathway). Ecotropic MLV particles are internalized into intracellular vesicles, but the vesicles are not colocalized with clathrin [75]. Furthermore, the dynamin-dominant negative mutant does not inhibit ecotropic MLV infection in human HeLa cells expressing the ecotropic MLV receptor, suggesting that ecotropic MLV particles are internalized by clathrin- and dynamin-independent endocytosis [75]. In contrast, another report indicates that siRNA-mediated knockdown of dynamin and dynasore suppresses ecotropic MLV infection in mouse NIH3T3, rat XC, and human TE671 cells expressing the ecotropic receptor [52] (Table 3). As mentioned above, the internalization pathway of ecotropic MLV might be dependent on the cell lines used. ALV [76] and EIAV [77] infections occur through clathrin-dependent endocytosis. JSRV infection required dynamin-dependent endocytosis [69]. Taken together, these reports strongly support a notion that infections by many animal retroviruses occur through endosomes and require endosome acidification.

All of intracellular vesicles do not necessarily become acidic. For example, macropinosomes can be recycled to plasma membrane before their acidification, and recycling endosomes are formed from early endosomes and are transferred to plasma membrane [48]. Because many retroviral infections require endosome acidification, if viral particles are internalized into recycling endosomes, infectivity would decrease. To prevent this, the interaction between retrovirus

Table 3: Differential internalization pathways of HIV and MLV infections.

Viruses	Internalization pathway	Cell lines	Reference
Ecotropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
	Dynamin-, clathrin independent	Human HeLa	[75]
Amphotropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
	Caveolin dependent	Mouse NIH3T3	[74]
Polytropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
Xenotropic MLV	Dynamin dependent	Mouse NIH3T3, human TE671, rat XC	[52]
CD4-dependent HIV	Dynamin dependent	Human HeLa	[95]
	Clathrin dependent	Human primary T lymphocyte	[95–97]
	Dynamin-, Eps15 dependent	Human HeLa	[98]
	Dynamin-, Eps15 independent	Human 293T, HeLa, TE671	[21]
CD4-independent HIV	Dynamin-, Eps15 dependent	Human 293T, HeLa, TE671	[21]

Env proteins and the infection receptors is speculated to induce a signal to trigger the acidification of virion-containing intracellular vesicles.

8. Cleavage of Retroviral Env Proteins by Cathepsins

Many retrovirus infections require endosome acidification. Influenza virus infection also requires endosome acidification, and treatment of influenza virus particles with low pH buffer activates its membrane fusion, indicating that low pH treatment directly induces conformational change of the influenza virus hemagglutinin to the fusion-active form. In contrast, low pH treatment of MLV particles does not activate the membrane fusion. Why does ecotropic MLV entry require endosome acidification?

There is another mystery of the endosome-mediated infection. Proteins internalized into acidic late endosomes/lysosomes are generally degraded by endosome proteases including cathepsins. The acidification inhibitors suppress the degradation in late endosomes/lysosomes [47]. If the retroviral particles are degraded in late endosomes/lysosomes, the acidification inhibitors would enhance retrovirus infection. However, the acidification inhibitors rather suppress the infection [52]. Therefore, it is suggested that the retroviral particles incorporated into late endosomes/lysosomes are not degraded. Why are the retroviral particles not degraded in acidic late endosomes/lysosomes?

The finding that endosomal cathepsin proteases are necessary for the ecotropic MLV infection [78, 79] like Ebola virus infection [80] has provided a clue to understanding the questions. Because cathepsin proteases are activated by acidification, the ecotropic MLV entry into host cytoplasm requires cathepsin activation by acidification. The weakly acidic condition (pH 6) in early endosomes cannot activate cathepsin proteinases [81], suggesting that ecotropic MLV infection occurs via late endosomes/lysosomes. The acidification inhibitors suppress MLV infections by attenuating cathepsin protease activation. The evidence that the acidification inhibitors do not suppress the ecotropic MLV infection in active cathepsin-containing medium further supports this conclusion [52]. Our current model for entry of ecotropic MLV is that cathepsin proteases digest MLV Env glycoproteins to generate fusion-active forms rather than to break them up completely, because treatment of ecotropic and amphotropic MLV particles with cathepsin B protease results in a few digested products of the Env proteins but not their disappearance [52, 79]. It is still unclear how the MLVs are not degraded in the late endosomes/lysosomes by other proteases.

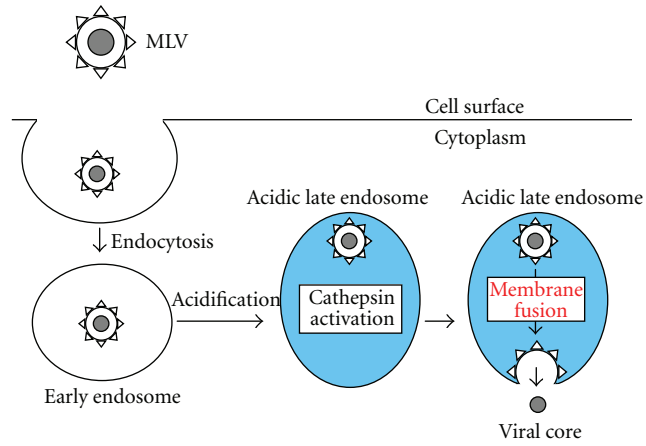

Figure 2: Entry pathway of ecotropic MLV in almost all susceptible cells. Blue area indicates acidic condition.

In summary, the entry pathway of ecotropic MLV occurs as follows (Figure 2). Ecotropic MLV particles are internalized into endosomes, following the interaction of Env protein with the infection receptor. The viral particle-containing endosomes become acidic by V-ATPase. Cathepsin proteases are activated in the acidic late endosomes. The activated cathepsins cleave the ecotropic Env proteins to confer them fusion active. The cleaved Env proteins induce fusion between the viral envelope and host cell endosome membranes. Finally, the ecotropic MLV cores enter into host cytoplasm.

Although it is widely accepted that the ecotropic MLV infection requires endosome acidification and cathepsin proteases, the entry pathway of xenotropic MLV is not clear,

because of the contradictory reports [52, 53]. We have shown that xenotropic MLV infection requires endosome acidification and cathepsin proteases like the ecotropic MLV infection [52]. In sharp contrast, the Liu research group has reported that inhibitors of endosome acidification and cathepsin proteases do not inhibit the xenotropic MLV infection [53]. Different cell lines used in these studies may induce different entry pathways of the xenotropic MLV.

Unlike the ecotropic MLV entry, it has been reported that a low-pH pulse of JSRV particles overcomes the bafilomycin-mediated infection inhibition [69], EIAV infectivity is enhanced by low-pH treatment [67], and cell-cell fusion induced by the ALV Env protein is enhanced at low pH [55]. Additionally, analysis of the pH dependence of the foamy virus Env-mediated fusion in a cell-cell fusion assay revealed an induction of syncytium formation by a short exposure to acidic pH [66]. The low-pH treatment of these retroviruses may directly induce the conformational changes of their Env glycoproteins to fusion active forms without the proteolytic cleavage, like influenza virus.

9. PH-Independent MLV Infection in XC Cells

Although the acidification inhibitors attenuate the ecotropic MLV infection in almost all susceptible cells [49, 52], the inhibitors have no effect on the ecotropic MLV infection specifically in rat XC cells, suggesting that the ecotropic MLV infection in XC cells is independent of low pH [49] (Table 2). Furthermore, the R peptide-containing ecotropic Env protein can induce pH-independent syncytium formation in XC cells, but cannot in other susceptible cells [82, 83]. By these results, it had been widely thought that ecotropic MLV entry into XC cells occurs at cell surface membranes and does not require the internalization of virions into intracellular vesicles and acidification. This XC cell-specific pH-independent ecotropic MLV infection was one of the well-known mysteries in the MLV field [84, 85]. We found that a cathepsin inhibitor, CA-074Me, efficiently suppresses the ecotropic MLV infection in XC cells, like in other susceptible cells, suggesting that the ecotropic MLV infection in XC cells requires endosomal cathepsin proteases [52]. This result is inconsistent with the previous theory that the ecotropic MLV infection in XC cells does not occur through endosomes. Because the ecotropic MLV infection requires cathepsin proteases activated by endosome acidification, the acidification inhibitors would be proposed to suppress the MLV infection by attenuating cathepsin activation. However, the acidification inhibitors do not reduce cathepsin activity in XC cells, but do so in other cell lines, suggesting that cathepsin proteases are activated without endosome acidification in XC cells [52]. XC cells do not express so much cathepsin that activation is sufficient at suboptimal pH, because cathepsin activity of XC cells is comparable to that of NIH3T3 cells. These results prompted us to speculate that the ecotropic MLV infection in XC cells occurs through endosomes. The result that dynasore and siRNA-mediated knockdown of dynamin expression suppress the ecotropic MLV infection in XC cells strongly supports this hypothesis.

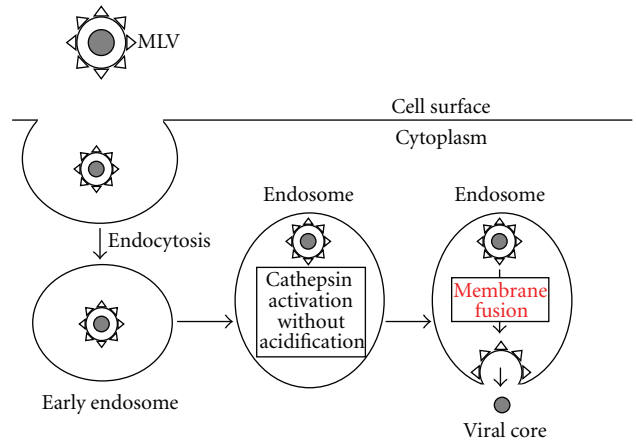

Figure 3: Entry pathway of ecotropic MLV in XC cells. Ecotropic MLV entry in XC cells may occur in acidic late endosomes, but endosome acidification is not required for the entry.

Taken together, the entry pathway of ecotropic MLV in XC cells is considered as follows (Figure 3). The ecotropic MLV particles are internalized into endosomes in XC cells, like in other susceptible cells. Cathepsin proteases are activated without endosome acidification. The activated cathepsins cleave the MLV Env protein, and the fusion between the viral envelope and host cell endosome membrane takes place for entry of the viral core into host cytoplasm. Because of the endosome acidification-independent activation of cathepsin proteases [52], the acidification inhibitors do not suppress the cathepsin protease activity and ecotropic MLV infection in XC cells. Additionally, this finding supports the above-mentioned hypothesis that the acidification inhibitors differentially affect retrovirus infections in different cell lines. The mechanism of acidification-independent cathepsin activation in XC cells is waiting to be resolved.

10. PH-Dependent Entry and PH-Independent Syncytium Formation by Retroviral Env Proteins

The R peptide-cleaved MLV Env protein induces the fusion between the viral envelope and host cell membranes for viral entry and syncytium formation in susceptible cells [33, 34]. Cells expressing the R peptide-truncated Env protein behave as large MLV particles and fuse with neighboring susceptible cells. Therefore, the syncytium formation by the retroviral Env proteins is thought to represent the membrane fusion in retroviral entry. Because the syncytium formation by the retroviral Env protein may contribute to the development of degenerative disorders like AIDS [28, 29], and because an endogenous retroviral Env protein (syncytin) induces syncytiotrophoblast formation [86], the elucidation of mechanism of retroviral Env-induced syncytium formation is essential to understand retroviral pathogenesis and placenta development. The MLV entry into host cells is dependent on low pH, but the syncytium formation by the R peptide-truncated Env protein is independent [33].

Furthermore, the viral envelopes fuse with host cell membrane in endosomes [52, 75], but the syncytium formation appears to result from the fusion of cell surface membranes of the Env-expressing and host cells. In addition, the Env glycoprotein of a CD4-independent HIV efficiently induces pH-independent syncytium formation [87], but infection by CD4-independent HIV occurs through acidic endosomes [21] (see below). Multiple interactions between the viral Env and infection receptor proteins in much larger areas of cell-cell contact than virus-cell contact may abrogate the requirement of endocytosis for the membrane fusion. The finding that a cell adhesion molecule, LFA-1, facilitates HIV-mediated syncytium formation but not HIV infection supports this idea [88]. If the syncytium formation by the Env protein is independent of endocytosis, cathepsin proteases would be unnecessary for the syncytium formation. However, cathepsin inhibitors suppress syncytium formation by the ecotropic MLV Env protein [79]. Secreted cathepsin proteases may be involved in the pH-independent syncytium formation by the Env protein. Further study is needed to understand the mechanism of pH-independent syncytium formation by the retroviral Env proteins.

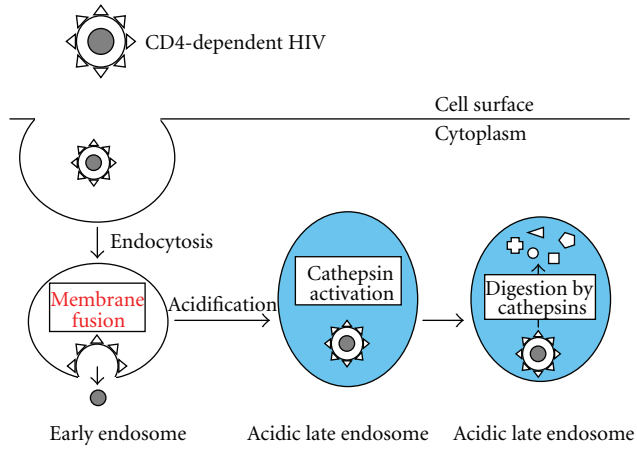

Figure 4: Entry pathway of CD4-dependent HIV. Blue area indicates acidic condition.

11. Endocytic Pathway of CD4-Dependent and -Independent HIV Entry

There are many controversial reports of the role of endocytosis in CD4-dependent HIV infection [94] (Tables 2 and 3). Early reports indicate that the acidification inhibitors enhance [89–91] or do not affect CD4-dependent HIV infection [92, 93], suggesting that the HIV does not enter into host cells via acidic vesicles. However, recent reports show that dynasore and chlorpromazine attenuate CD4-dependent HIV infection [95–97]. In addition, dominant negative mutants of dynamin and Eps15 inhibit CD4-dependent HIV infection [98]. Furthermore, analysis of localization of labeled HIV particles revealed that the HIV particles are internalized into intracellular vesicles [95, 99–102]. It has been reported that envelopes of HIV particles fuse with host cell membranes in intracellular vesicles by the following observation [95]. Envelopes of HIV particles were labeled with a hydrophobic fluorescent compound. When fusion of the labeled HIV envelope with host cell membrane occurs, the fluorescent compound is diluted and the fluorescent signals disappear. The vanishing of the fluorescent signals was observed in the intracellular vesicles but not at cell surfaces. These results suggest that HIV entry into the host cell cytoplasm may occur via endosomes.

Interestingly, endosome acidification inhibitors attenuate infections by CD4-independent HIVs, which are thought to be prototypes of CD4-dependent viruses, suggesting that CD4-independent HIV entry may occur through acidic late endosomes, like many animal retroviruses [21]. The CD4-dependent HIVs can infect CD4-negative trophoblastic cells though the infection is 100 times less efficient than CD4-dependent Env-mediated infection [103]. HIV infection of trophoblasts forming the placental barrier may cause the mother-to-child transmission of HIV [104]. This infection occurs through an unusual entry pathway that is clathrin-, caveolin-, and dynamin-independent endocytosis requiring free cholesterol [71].

12. Degradation of HIV Particles by Endosome Proteases

Because acidification inhibitors enhance CD4-dependent HIV infection [89–91], HIV entry is independent of low pH, and the viral particles internalized into acidic late endosomes are degraded [105]. In other words, a proportion of HIV particles are internalized into acidic late endosomes although the internalization into late endosomes is not associated with the HIV productive infection. Consistently, the HIV particles appear to be internalized into acidic compartments shortly after inoculation into host cells [100].

In summary, entry pathway of CD4-dependent HIV is considered as follows (Figure 4). The HIV particles are internalized into host cells by endocytosis, and the entry is independent of endosome acidification. HIV entry mainly occurs at early endosomes, and the HIV particles internalized into acidic late endosomes are degraded by endosome proteases.

It has been reported that a cathepsin inhibitor CA-074Me more significantly enhances CD4-independent HIV infection than CD4-dependent infection, and cathepsin protease activity in host cells is reverse-correlated with cellular susceptibility to the CD4-independent HIV infection [21]. These results suggest that CD4-independent HIV entry may occur at acidic late endosomes, and that viral entry competes with virion degradation by cathepsin proteases (Figure 5).

Degradation by endosomal proteases in acidic vesicles following phagocytosis/macropinocytosis/endocytosis functions as an innate immune reaction against microbes to digest them and generate antigen peptides presented to helper T cells on MHC class II [106]. In fact, the activation of toll-like receptor signaling by LPS enhances cathepsin expression [21]. The CD4-dependent HIVs might evolve

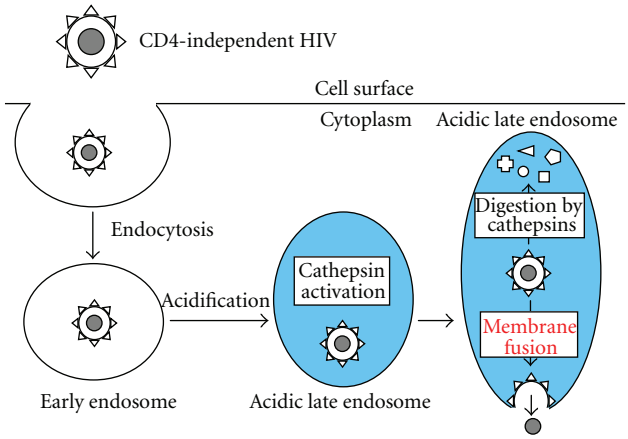

Figure 5: Entry pathway of CD4-independent HIV. Blue area indicates acidic condition.

from CD4-independent viruses to overcome the endosome protease-mediated immunity. Some microbes express cystatin-like cathepsin inhibitors to protect themselves from the cathepsin-mediated immunity [107, 108]. Instead of having a cathepsin inhibitor, the CD4-dependent HIVs might gain the acidification-independent entry mechanism to protect from the endosome protease-mediated immunity.

In contrast to the CD4-dependent HIV entry pathway, ecotropic MLVs utilize these cellular innate immune reactions of endocytosis, acidification, and digestion by endosome proteases to enter into the host cell cytoplasm. By the ecotropic virus entry mechanism, the viruses can escape from these host immune reactions. It is suggested that the CD4-dependent HIV entry utilizes endocytosis, but not acidification and proteolysis by endosome proteases. The CD4-dependent HIV particles may be degraded by endosome proteases in acidic endosomes, and the infection titer is reduced [89, 91]. The CD4-dependent HIV Env proteins indeed contain several amino acid motifs that are digested by cathepsins [109, 110]. The ecotropic MLVs also have cathepsin-recognized amino acid motifs, but the digestion may activate the membrane fusion capability of the Env protein.

As mentioned above, the cathepsin inhibitor enhances CD4-independent HIV infection in cells with relatively higher level of cathepsin protease activity [21]. While, treatment of such cells with CA-074Me at higher concentration attenuates the CD4-independent infection. In addition, CA-074Me suppresses the CD4-independent HIV infection in cells with lower cathepsin activity (unpublished data). These results suggest that cathepsin proteases are required for the CD4-independent infection. Therefore, Env glycoproteins of the CD4-independent HIVs may be digested by cathepsin proteases to a fusion-active form, like the ecotropic MLV Env protein. Consistently, cathepsin proteases enhance CD4-dependent HIV infection and confer CD4-negative cells susceptible to CD4-dependent HIV infection [111–113]. Cathepsin-mediated digestion of CD4-dependent HIV Env protein may induce membrane fusion without CD4 binding. HIV particles in acidic endosomes are degraded by many endosome proteases including cathepsins. However, when the HIV Env proteins are digested only by a cathepsin, the infectivity may be enhanced.

13. Entry of Targeted Retroviral Vector

Retroviral vectors are valuable tools in molecular biology research and human gene therapy. Several fundamental properties of retroviral vectors remain to be improved for effective gene transfer to specific target cells [114]. The effectiveness will be greatly enhanced, if their infection tropism is artificially modified to target specific cells [115]. There have been various attempts to establish redirecting infection tropism by genetically incorporating heterogenous ligands into the retroviral Env proteins [116–121]. However, retroviral vectors containing such modified Env proteins suffer from very low transduction efficiency or are not infectious. The redirected transductions of retroviral vectors with chimeric Env proteins are enhanced by the endosome acidification inhibitors, suggesting that the targeted vector particles internalized into acidic endosomes are degraded by endosome proteases [120, 122].

Retroviral vectors carrying the ecotropic Env proteins chimeric with SDF-1α [123] and somatostatin [124] can transduce cells expressing CXCR4 and somatostatin receptor, respectively, as efficiently as retroviral vectors with the wild-type Env protein. It has not been examined whether efficient infections by the redirected retrovirus vectors occur through endosomes. Because the SDF-1α-chimeric Env protein appears to induce infection by the same mechanism as the wild-type Env protein [125], the redirected infection may occur through endosomes and require endosome acidification, like the wild type MLV Env protein. Elucidation of the entry pathways of these targeted retroviruses will likely contribute to the development of efficient cell lineage-specific retrovirus vectors.

14. Endocytic Entry of Ebola Virus-Pseudotyped Retrovirus Vector

Retrovirus vectors can be pseudotyped with glycoproteins of various enveloped viruses. The pseudotyped retrovirus vectors enter into host cells by the entry mechanisms of the heterologous viral glycoproteins. Because the retrovirus vectors do not produce replication-competent viruses and the protocol is relatively simple, pseudotyped retrovirus vectors are widely used to identify entry pathways of various enveloped viruses [126–128].

A dominant negative mutant of Eps15, siRNA-mediated knockdown of clathrin, and chlorpromazine suppress infection by an HIV vector pseudotyped with Ebola virus glycoprotein (GP), indicating that Ebola virus GP-mediated entry occurs through clathrin-dependent endocytosis [129]. Virion morphologies of the pseudotyped HIV vector and Ebola virus are much different. The pseudotyped HIV vector particles are round and the diameter is around 100 nm regardless of viral envelope glycoproteins. Whereas Ebola

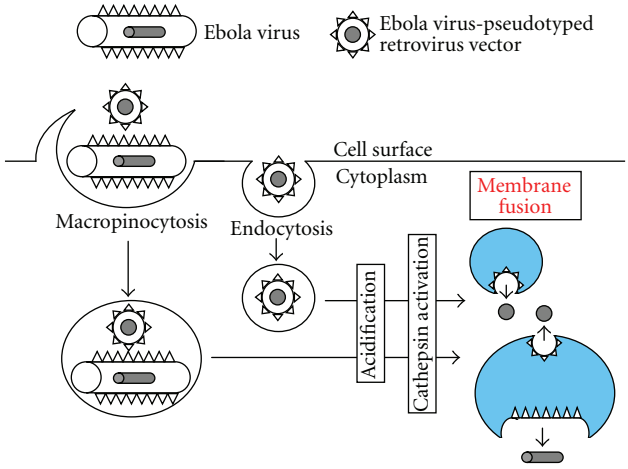

Figure 6: Entry pathways of Ebola virus and Ebola virus-pseudotyped retrovirus vector. Blue area indicates acidic condition.

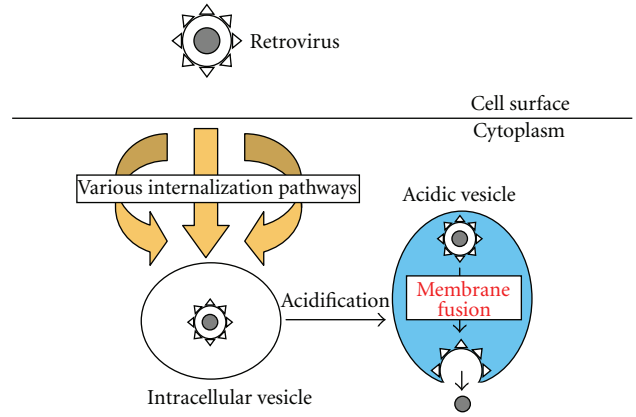

Figure 7: Retrovirus particles are internalized into intracellular vesicles by various pathways, and vesicle acidification is necessary for the infections.

virus virions are long and filamentous as the name of filovirus should show. Typical clathrin-coated vesicles are large enough to incorporate the HIV vector particles, but not Ebola virus particles. Therefore, Ebola virus particles cannot be internalized into the endosomes. Does Ebola virus enter into host cells through endosomes? The finding that Ebola virus entry occurs via macropinosomes resolved this problem [130–133] (Figure 6). Macropinosomes have enough size to incorporate Ebola virus particles. However, entry of intact Ebola virus is still dependent on dynamin, which is not involved in classical macropinocytosis [133], and is partially inhibited by inhibitors of clathrin-dependent endocytosis [132]. In addition, it has been reported that the Ebola virus entry through macropinocytosis or endocytosis is dependent on the cell lines used [134]. Therefore, the entry route of Ebola virus is not clear yet. The Ebola virus infections via endocytosis and macropinocytosis both require acidification and cathepsin proteases [80, 135]. Although the pseudotyped retrovirus vector is useful to study the entry mechanism of viral envelope proteins, we should notice the possibility that entry pathway of the pseudotyped retrovirus vector is different from that of the original virus.

Size of macropinosomes is enough to incorporate not only Ebola virus particles but also pseudotyped HIV vector particles. Therefore, Ebola virus-pseudotyped HIV vector entry can occur through macropinocytosis (Figure 6). There is a report showing that HIV infection occurs through macropinosomes [102]. If host cells have both dynamin-independent macropinocytosis and -dependent endocytosis, the inhibition of dynamin function does not significantly affect the pseudotyped HIV vector infection. If host cells have endocytosis but not macropinocytosis, the inhibition of dynamin function severely suppresses the pseudotyped HIV vector infection. Retrovirus entry may be able to occur through several distinct internalization pathways for productive infection (Figure 7). This may be the reason why the inhibitors differentially affect retrovirus infections in different cells. Pathways of retrovirus internalization into intracellular vesicles may be unimportant for the productive infection. The GP of Ebola virus that enters host cells via macropinosomes can use endocytosis for the productive entry, when the retrovirus vector is pseudotyped with the Ebola virus GP. This result strongly supports the idea.

15. Conclusion

Infections by many animal retroviruses occur through endosomes and require endosome acidification. The activation of cathepsin proteases by endosome acidification is required for ecotropic MLV infection. Whereas acidification directly induces conformational changes of several retroviral Env proteins to the fusion active forms. There are several internalization pathways of retrovirus particles, and the viral internalization pathways appear to be different in different cell lines. CD4-independent HIV infection may occur through endosomes and require endosome acidification, like other animal retroviruses. CD4-dependent HIV infection is thought to occur through endosomes but does not require endosome acidification. The CD4-dependent and -independent HIV particles are both degraded by endosome proteases, when the viral particles are internalized into acidic late endosomes. Retrovirus vectors pseudotyped with other viral envelope proteins are widely used to understand the entry mechanisms of the envelope proteins. However, entry pathway(s) of the pseudotyped retroviral vector could be different from that of the original virus.

Retroviruses require cellular biological events of internalization, vesicle acidification, and cathepsin proteolysis for their entry into host cells. These biological events, especially in phagocytosis, function to protect host cells from microbe infection. Retroviruses utilize these immune reactions to enter into host cells. This entry mechanism of retroviruses is the best strategy to overcome the host immune attack, and many viruses other than retroviruses also enter into host cells by similar mechanisms [72, 136].

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Review Article

Endocytosis of Integrin-Binding Human Picornaviruses

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Picornaviruses that infect humans form one of the largest virus groups with almost three hundred virus types. They include significant enteroviral pathogens such as rhino-, polio-, echo-, and coxsackieviruses and human parechoviruses that cause wide range of disease symptoms. Despite the economic importance of picornaviruses, there are no antivirals. More than ten cellular receptors are known to participate in picornavirus infection, but experimental evidence of their role in cellular infection has been shown for only about twenty picornavirus types. Three enterovirus types and one parechovirus have experimentally been shown to bind and use integrin receptors in cellular infection. These include coxsackievirus A9 (CV-A9), echovirus 9, and human parechovirus 1 that are among the most common and epidemic human picornaviruses and bind to αV-integrins via RGD motif that resides on virus capsid. In contrast, echovirus 1 (E-1) has no RGD and uses integrin $\alpha 2\beta 1$ as cellular receptor. Endocytosis of CV-A9 has recently been shown to occur via a novel Arf6- and dynamin-dependent pathways, while, contrary to collagen binding, E-1 binds inactive $\beta 1$ integrin and enters via macropinocytosis. In this paper, we review what is known about receptors and endocytosis of integrin-binding human picornaviruses.

1. Introduction

Picornaviruses (family *Picornaviridae*) include a diverse group of viruses, arguably best known not only as causes of devastating acute human (polio) and animal diseases (foot and mouth disease) but also by as the most common infectious disease, common cold, caused by human rhinoviruses, aseptic meningitis caused by coxsackieviruses, and more recently by severe CNS infections of newborns caused by human parechoviruses [1–4]. Clinical manifestations of picornaviruses are variable, including respiratory symptoms, gastroenteritis, rash, myocarditis, neonatal sepsis-like disease, and infections of the central nervous system such as acute flaccid paralysis, meningitis, and encephalitis [5–8]. Some virus types have also been linked to chronic diseases such as type 1 diabetes mellitus [9, 10] and wheezing illnesses that may develop into asthma [11, 12]. In spite of clinical importance of picornaviruses, there are no approved drugs against them, and the only vaccines are against three poliovirus types and hepatitis A virus [2].

Currently, there are twelve genera with 29 species in the family *Picornaviridae*. Six genera contain virus types that infect humans (*Enterovirus*, *Cardiovirus*, *Aphthovirus*, *Parechovirus*, *Hepatovirus*, and *Kobuvirus*) [13, 14]. The genus *Enterovirus* contains most of the human picornaviruses, with more than 250 recognized types, while the genus *Parechovirus* contains 16 recognized types based on VP3/VP1 sequence analysis (http://www.picornaviridae.com/). Since many of the newly discovered entero- and parechoviruses have been identified only by genetic methods [15], there is no purified virus material available for experimental studies. Thus far most picornaviral studies have been performed using a limited number of model viruses (such as poliovirus, echovirus 1, coxsackievirus B3, and human rhinovirus 2), and, hence, we know next to nothing of life cycle of other picornaviruses. Life cycle of most human viruses is dependent on efficient entry through plasma membrane into cell cytosol in endosomal vesicles, which transport the viruses to the site of replication in the cell interior. Internalization of many viruses involves classical clathrin-mediated

endocytosis. However, our understanding on endocytic routes has been challenged during the past decade by introduction of novel pathways including macropinocytosis and caveolar/lipid raft-mediated endocytosis and other pathways of which we have rather limited information. During the recent years, the area of (viral) endocytosis has been the subject of extensive reviewing [16–29]. Whereas many viruses that use integrins as their cellular receptors are internalized in clathrin-coated pits and later found in endosomes [30–32], some integrin-binding picornaviruses seem to use alternative entry pathways. The aim of this paper is to give an overview of what we know about receptors and endocytosis of integrin-binding human picornaviruses. Although limited in number, they include virus types that are epidemic and pathogenic. Interestingly, these viruses share common features, which are not reflected in their receptor use and mechanism of endocytosis. Coxsackievirus A9 (CV-A9) and human parechovirus 1 possess RGD motif through which they bind αV-integrins. However, they seem to prefer different integrin receptors in cellular entry, and the endocytic route is also different. CV-A9 seems to endocytose via a novel Arf6- and dynamin-dependent pathway, which has not previously been described for viruses. Contrary to the expected, echovirus 1 binds inactivated integrin and is capable of alleviating the natural signaling mechanism in cell entry. Studies on virus receptors in general may give clues on viral endocytic routes and therefore enable understanding of the mechanisms of virus receptor-mediated endocytosis. This will allow the identification of the common key molecules that mediate virus life cycle and help in development of antivirals against picornaviruses.

2. Virus Structure, RGD Motif, and Integrin-Binding Picornaviruses

Picornaviruses are small icosahedral viruses composed of three or four capsid proteins (VP1-4 or VP0, VP1, and VP3 in the case of human parechoviruses, which lack proteolytic processing between VP4 and VP2) [33, 34]. While VP1-3 forms the icosahedral shell, VP4 protein resides in the inner surface and is probably in contact with a single species of single-stranded, plus-sense viral RNA genome, which is approximately 7000–8500 nucleotides long. RNA organization is fairly similar across the virus family [14]. A single polyprotein is produced from the RNA genome, which is proteolytically cleaved into mature structural (capsid) and nonstructural proteins. Capsid monomers assemble into pentamers, which form the complete icosahedral shell of the virus with twelve pentamer subunits. The structure of monomers and their spatial location determine the surface topology of the virus particle and are responsible for receptor binding. VP1 is the most important protein in receptor binding. Enteroviruses possess a deep depression, "canyon," in the fivefold axis below VP1, which is involved in receptor interactions [35, 36]. The canyon has been targeted with small molecular compounds to develop antivirals, and several compounds have been shown to bind to the canyon and inhibit virus infection [37, 38]. Disappointingly, none of the compounds is in clinical use. The first structure of human parechovirus was only recently elucidated in complex with integrin receptor, and the coordinates for receptor binding were similar to enteroviruses [39]. In all, high-resolution structures of virus-receptor complexes have been determined for only a few picornaviruses, and there is a clear lack of knowledge regarding virus-receptor binding, and subsequent endocytic events.

More than ten primary cellular receptors have been implicated in picornavirus infections, and many of them belong to the immunoglobulin superfamily [2, 40, 41]. One group of such picornaviral receptors are integrins, which form a family of transmembrane glycoproteins that form noncovalent heterodimers with eighteen α- and eight β-integrin subunits that combine to form 24 $\alpha\beta$ receptors [42]. Besides picornaviruses, integrins are optimal cellular receptors for adeno-, rota-, hanta-, and herpesviruses [43] because they are abundantly expressed on most cell types (see integrins at http://www.signaling-gateway.org/). In addition, integrins are connected to signalling proteins that may trigger endocytic pathways. They regulate a wide variety of cellular processes such as cell migration, growth, phagocytosis, inflammation, wound healing, and neoplasia, and they also participate in adhesive events including haemostasis and thrombosis [44]. The long extracellular domains of integrins interact with various extracellular matrix (ECM) proteins (laminin, collagen, vitronectin, and fibronectin) and other ligands including viruses, while the short cytoplasmic domains of integrins interact with the actin cytoskeleton and its components. Thus, the functional domains of integrin link the external side of the plasma membrane to the internal side of the cells to mediate internalization and endocytosis of cargo molecules [2, 45, 46]. Activation of integrin-mediated signalling is considered to be an essential mechanism for the internalization of viruses. In this process, they may mimic the natural ligands. The mechanisms regulating virus entry seem to significantly overlap with the known endocytic pathways for integrins and may, therefore, provide important insights into the mechanisms regulating integrin traffic. However, recent findings indicate that binding and internalization of some viruses such as echovirus 1 to integrin receptor differ from that of natural ligand (see below) indicating that viruses are capable of using alternate entry pathways. Integrin-binding picornaviruses also use other cell surface molecules for binding and/or entry into cells. Currently, the actual function of both integrins and these other molecules in virus infection is largely unknown, and it remains to be shown whether they are simply attachment receptors or mediators of virus internalization [2].

Out of more than one hundred enterovirus and sixteen human parechovirus types [13], there are around twenty model viruses for which there is experimental evidence for receptor binding and/or entry route [2]. Three enterovirus types (coxsackievirus A9 (CV-A9), echovirus 9 (E-9), and echovirus 1 (E-1), and a single parechovirus type (human parechovirus 1 (HPeV-1)) have been experimentally shown to bind to integrin receptors [39, 47, 48]. With the exception of E-1, they possess a specific RGD (arginine-glycine-aspartic acid) motif for integrin binding [49]. RGD-binding integrins include five αV integrins (αVβ1, αVβ3, αVβ5, αVβ6, and

$\alpha V\beta 8$), two $\beta 1$ integrins ($\alpha 5\beta 1$ and $\alpha 8\beta 1$), and $\alpha IIb\beta 3$ and share the ability to recognize cellular ligands such as fibronectin and vitronectin via RGD motif [50]. CV-A9 and HPeV-1 have been shown to bind *in vitro* to $\alpha V\beta 3$ and $\alpha V\beta 6$ [39, 47, 51], and there is experimental evidence for the binding of E-9 to integrin $\alpha V\beta 3$ [48]. In addition, there are other proteins that contribute to cellular infection of these viruses, which complicate the analysis of their role in viral endocytosis. E-1 is non-RGD enterovirus, which binds to integrin $\alpha 2\beta 1$. Besides E-1, there is a single report claiming that several other non-RGD echoviruses bind to integrin $\alpha V\beta 3$ [52], but the results have not been confirmed by other groups.

3. *In Silico* Analysis of RGD Motif on Viral Sequences

Analysis of all picornaviral sequences in the GenBank (on 26.6.2012) with the scan for protein motif method implemented in DAMBE (http://dambe.bio.uottawa.ca/software.asp) revealed novel picornaviruses that contain RGD motif(s), and which may thus use integrin receptors in cellular entry (Table 1). RGD motifs were identified in four types of *Human enterovirus B* species and in one type of *Human enterovirus C*. There were no RGDs in the sequences of virus types in *Human enterovirus A* and *D* species. Among the eight parechovirus sequences analyzed, an RGD motif was identified in five of them (HPeV-1, 2, 4, 5, and 6). This motif was found in similar locations in the flexible C-terminus of VP1 protein in CV-A9, E-9, EV-B83, and EV-B99. Although the C-terminal 15 amino acids of VP1 containing the RGD motif could not be identified in the X-ray structure [53], the structural coordinates for integrin binding to CV-A9 and HPeV-1 suggest that this site may be functional in integrin binding, and therefore all of these viruses may bind to integrin receptors via an RGD motif [39]. Structural comparison of other putative RGD sites to the structure of human echovirus 7 (E-7; PDB id: 2x5i) with Discovery Studio software (Discovery Studio Modeling Environment, Release 3.1, San Diego: Accelrys Software Inc., 2011) suggested that they are surface-exposed. However, further experimentation is needed to elucidate their role in integrin binding. In all, there may be in total of four enteroviruses and five parechoviruses, which possess RGD motif in the C-terminus of VP1 protein and may therefore bind integrin receptor(s) (Table 1).

Table 1: RGD motif(s) identified in picornaviral capsid proteins.

Type	Acc no.	Location of RGD motif in viral polyprotein	
		VP3	VP1
CV-A9	D00627		858–860
E-5	AF083069	410–412	
E-9	X84981		651–653/860–862
EV-B83	AY843301		832–834
EV-B99	JF260926		860–862
HPeV-1	L02971		764–766
HPeV-2	AJ005695		763–765
HPeV-4	DQ315670		767–769
HPeV-5	AF055846		772–774
HPeV-6	AB252582		766–768

4. Mechanisms of Endocytosis of Integrin-Binding Human Picornaviruses

4.1. Echovirus 1. One of the most studied picornaviruses in respect to integrin receptor binding and endocytosis is echovirus 1 (E-1). While the other integrin-binding human picornaviruses bind αV-integrins via an RGD motif near the C-terminus of VP1, E-1 is exceptional in that it is the only known picornavirus to bind integrin $\alpha 2\beta 1$ [54]. Integrin $\alpha 2\beta 1$ (also known as Ia-IIa, VLA-2, ECMR-II) is a collagen receptor, which is expressed in fibroblasts, platelets, and in endothelial and epithelial cells [55]. The primary binding site of E-1 in integrin $\alpha 2\beta 1$ is the inserted domain in the $\alpha 2$ subunit ($\alpha 2$I) [56–58]. The binding site of E-1 overlaps with binding site of collagen thus preventing collagen interaction with $\alpha 2$I domain when virus binds to integrin [54, 59]. E-1 binds to that domain ten times more efficiently than collagen [54]. In a cryo-EM analysis, the virus particle appeared to be decorated with 60 copies of the integrin $\alpha 2$I domain suggesting that each VP1-3 protomer can bind one $\alpha 2$I domain. The $\alpha 2$I domain interacts with the VP2 of one protomer and the VP3 of a neighbouring protomer in the E-1 capsid [54]. Binding of E-1 to integrin $\alpha 2\beta 1$ does not induce uncoating but instead may lead to the stabilization of capsid suggesting that viral RNA is released during endocytosis and not on plasma membrane [37, 46]. Unlike the cellular ligand, collagen, which binds active, extended form of the receptor with subsequent elicitation of integrin-mediated signalling cascades and endocytosis, E-1 was recently shown to prefer bent form of $\alpha 2\beta 1$ in binding [60, 61]. Virus binding does not lead to activation of the mechanism of cellular endocytosis but instead the virus clusters integrins, which seems to be essential for rapid internalization of the virus [60, 61]. Thus, E-1 is targeted to a novel, $\beta 1$-mediated endocytic pathway, but the mechanism remains unclear.

The initial observations of cellular entry of E-1 suggested that the virus uses caveolar/raft-dependent entry pathway. This was based on the virus accumulation in caveolin-1-positive endosomes in SAOS cells overexpressing integrin $\alpha 2\beta 1$ [45, 46]. However, at the same time and using another cell model, CV-1, the same authors demonstrated that majority of E-1 do not colocalize with caveolin-1 on the plasma membrane [54]. This observation was based on parallel comparisons to SV40, which is known to use caveolar route at least in some cell lines [47]. However, it is now evident that while some E-1 particles accumulate in caveolin-1-positive vesicles, the internalization mechanism of E-1 has features of macropinocytosis since many signalling molecules that are involved in E-1 infection are macropinocytosis-linked [16, 62]. These molecules include PKCα, PLC, PI3 K, Rac1, Pak1, and CtBP1/BARS [60, 61]. Caveolar pathway was originally characterized by structural proteins of caveosomes,

caveolin-1, and caveolin-2 and lacks typical markers of clathrin-mediated endocytosis such as epsin family proteins and clathrin. Dynamin has been shown to be indispensable in caveolar pathway [23, 63]. However, it has been suggested that caveolar, lipid raft-dependent, and caveolin-1-independent endocytic pathways have the same underlying mechanisms [64]. In addition, the view on caveosomes has recently been revised in that they correspond to modified late endosomes or endolysosomes, and it has been suggested that the term caveosome is no longer to be used [65].

More recent studies indicate that in the early stage of endocytosis, E-1 is found in tubulovesicular structures, which contain fluid-phase markers but do not contain caveolin-1, GPI-anchored proteins, or flotillin. This suggests that E-1 entry pathway originates from lipid rafts and is not linked to caveolar or flotillin pathways. Studies on α2-positive structures indicated that α2/E-1 is internalized in pH-neutral vesicular bodies, which lack the features of clathrin-mediated endocytosis. Yet, E-1 was also detected in caveolin-1-positive structures after 15 minutes postinfection [66–68] and dominant-negative caveolin-3 has been shown to block E-1 infection [52]. These data suggest that caveolar pathway is somehow linked to E-1 endocytosis. The novel structures that contain E-1 and internalized integrins are called α2 integrin containing multivesicular bodies (α2-MVBs) [60, 61, 68, 69]. The α2-MVBs are degradative structures distinct from lysosomes, autophagosomes, and proteasomes [69, 70]. The nature of α2-MVBs involved in E-1 entry was confirmed by using immunofluorescence and siRNAs against components of endosomal sorting complex of transport proteins (ESCRTs), which are localized in early endosomes and function in MVB formation [55]. Their role in E-1 endocytosis is still unclear but they may prevent the contact between E-1 and lysosomal hydrolases, which would be detrimental for successful E-1 infection [69, 70]. The recent finding that ESCRT complex recruits caveolin-1 into maturing intralumenal vesicles may explain why E-1 and caveolin-1 are found in similar structures early in infection [46]. After internalization, clustered integrins are not recycled back to plasma membrane in strict contrast to slow recycling of unclustered α2 integrins [70]. This may indicate that E-1 has means to reroute endocytic vesicles, which contain α2 integrins. Internalization of β1 integrin has been shown to be regulated by Rab5/Rab21 and microtubules [43] as well as active Arf-6 [71]. However, the role of Arf-6 in viral endocytosis ([72]; see below) was only recently demonstrated, and such studies have not been performed with E-1.

4.2. Echovirus 9 and Coxsackievirus A9. Echovirus 9 (E-9) and coxsackievirus A9 (CV-A9) are structurally very similar viruses. They possess a receptor-binding motif, RGD, at the C-terminus of the VP1 protein through which they interact with cell surface integrin(s). They are epidemic viruses and among the most common etiological agents of aseptic meningitis [73–76]. Two known strains of E-9, Barty and Hill, differ in that Barty strain possesses RGD motif while Hill does not. The lack of RGD severely affects the infectivity of the Hill strain [48], and it has been speculated whether Hill is a true strain or instead, a recombinant form between Barty strain and E-18 [77]. RGDless E-9 viruses have not been detected in epidemiological samples, and, therefore, it is likely that RGD motif for integrin binding is imperative for successful E-9 infection [48]. Since there are no imaging studies on endocytosis of E-9, it remains to be determined whether E-9 follows the same route as CV-A9 since the viruses are very similar in sequence and pathogenicity [53]. With this in mind, the remaining part of this chapter will focus on receptors and endocytosis of CV-A9.

Although a number of cell surface molecules have been proposed to contribute to CV-A9 infection, the internalization mechanism that follows after the virus particle has bound to cell surface is still poorly characterized. CV-A9 has been shown to interact *in vitro* with integrin αVβ3 and αVβ6 via RGD motif [51, 78, 79]. RGD motif seems to be essential in clinical CV-A9 infections since it is conserved in CV-A9 isolates [80]. However, Hughes et al. [81] and Roivainen et al. [82] showed that artificial CV-A9 mutants lacking the RGD motif efficiently infected human rhabdomyosarcoma (RD) cells, indicating that there are also an RGD-independent attachment and an internalization mechanism. In addition, there are cell lines that do not express αV-integrins but which are highly susceptible to infection with CV-A9 [83]. Triantafilou et al. [84] showed that CV-A9 binds to Chinese hamster ovary (CHO) cells that overexpress human integrin αVβ3, although this interaction does not lead to productive cell infection. More recently, it has been shown that integrin αVβ6 binds with higher affinity to CV-A9 than integrin αVβ3 suggesting that it is the primary cellular receptor for CV-A9 [47]. The binding to αVβ6 was recently shown to occur at nanomolar affinity but there were no indications of structural changes that would lead to RNA release [85]. Besides integrin(s), there are other cellular molecules that participate in the entry stage of CV-A9 infection. Triantafilou et al. [84] were the first to demonstrate that antibody against β2-microglobulin (a subunit of major histocompatibility complex class I (MHC-I) complex β2M) inhibited CV-A9 infection completely in susceptible cell lines. In addition, heat shock 70-kDa protein 5 (HSPA5; also known as glucose-regulated protein 78-kDa, or GRP78) has been shown to mediate CV-A9 infection possibly via its interaction with β2M [86]. Recent report suggests that some CV-A9 isolates bind heparan sulphate [87].

Endocytosis of CV-A9 has mainly been followed in green monkey kidney (GMK) and human lung carcinoma (A549) cell lines [72, 88, 89]. Hecker et al. [88] demonstrated in their early work by electron microscopy that CV-A9 particles enter GMK cells in vesicles, which then occasionally fuse and form larger structures. It was also found that most internalized virus particles became trapped in large vacuoles (presumably lysosomes) where they were confined without proceeding to capsid uncoating and RNA release. In GMK cells, the entry of CV-A9 was proposed to occur through lipid microdomains where a number of signalling events takes place [89]. Other enteroviruses, for example E-1 [66, 67] and E-11 [90], have also been shown to utilize lipid microdomains for cellular entry. However, lipid rafts are involved in several entry pathways and, therefore, are not very indicative of cell entry route [20]. A more detailed study on CV-A9 endocytosis was

conducted in A549 cells using siRNAs and confocal imaging [72]. Contrary to expected, silencing of Src, Fyn, RhoA, PI(3)K, and Akt1 that have been linked to integrin signalling had no effect on CV-A9 infection. In addition, CV-A9 did not cause integrin receptor clustering as in the case of E-1 and $\alpha 2\beta 1$. We recently showed [72] that CV-A9 internalization is dependent on β2-microglobulin [75]. CV-A9 was internalized but retained at the cell periphery following the silencing of β2M. This was the first visual demonstration that β2M functions at postinternalization stage. These data suggest that integrin $\alpha V\beta 6$ acts merely as binding receptor while the internalization is mediated by β2M.

The current view on virus endocytosis is based on specific cellular markers that are used to distinguish between the endocytic routes. However, many central markers have been found to overlap between these routes. For example, dynamin was originally noted for its role in severing clathrin-coated vesicles from plasma membrane, but was then found to be indispensable for example in caveolar and some other clathrin-independent pathways [24, 71, 91]. The role of Arf6 in virus endocytosis is also becoming increasingly important. Arf6 (ADP-ribosylation factor 6) is a small GTPase, which has multiple roles in the regulation of membrane traffic and other cellular functions, but it was only recently when it was linked to virus endocytosis [75]. Although it has been suggested in the current classification schemes that Arf6-dependent entry pathway is dynamin-independent [20], it has been shown that structural protein VP22 of Herpes simplex virus uses both Arf6 and dynamin in the internalization process in HeLa cells. The entry process of HSV-VP22 appears to be similar to that of CV-A9 since internalization of HSV-VP22 is independent of clathrin, caveolin, and the Rho family GTPases RhoA, Rac1, and Cdc42 [92]. Our recent data indicate that CV-A9 entry, irrespective of RGD, is dependent on β2M, HSPA5, dynamin, and Arf6, and that CV-A9 and Arf6 are coendocytosed, that is, they are localized in the same vesicles when being transported from cell surface to the interior [72, 83, 84, 86]. Interestingly, Arf6 also regulates the endocytosis of MHC-I proteins [93] including β2M, which suggests a possible mechanistic link between these molecules and therefore internalization of CV-A9.

One of the many unanswered questions regarding CV-A9 infection is the role of integrin and other putative receptors play in cellular entry. Conceptually, there are attachment receptors that bind viruses to concentrate them on the cell surface, and actual virus receptors, which in addition to their role as binding receptors trigger changes in the virus structure to potentiate RNA release and induction of cellular signalling that mediates virus internalization. It is evident that integrin $\alpha V\beta 6$ serves as high-affinity binding receptor for CV-A9, while at least two other molecules mediate the internalization and possibly cellular endocytosis of the virus in cell-specific manner. In a simplified model, CV-A9 particle binds first to the cell surface integrin $\alpha V\beta 6$ (in RGD-dependent endocytosis) and then rapidly forms association with β2M (and HSPA5). Following this, the virus is endocytosed via an Arf6-mediated route, perhaps still in association with β2M. However, the conserved nature of RGD motif in clinical CV-A9 isolates indicates that integrin binding must have importance in multicellular infection. It is possible that CV-A9 is capable of altering receptor use at organism level while maintaining its infectivity by existing in quasispecies form, and this may explain why it remains highly pathogenic [63]. There are also reports claiming that β2M participates in entry stage of several echoviruses including E-1 [56, 94] and possibly human parechovirus 1 (see below). The MHC I/β2M is linked to integrin-mediated cellular endocytosis and may serve as cue to reroute viruses into novel locations within endosomal network. Endocytosis of MHC I has been studied in detail in HeLa cells, and it is visible in endosomes that are distinct from those containing cargo, such as transferrin receptor that enters by clathrin-mediated endocytosis. At later time points (10–15 min), MHC I is observed in classical early endosomes containing the transferrin receptor, Rab5, and the early endosomal antigen 1 (EEA1). EEA1 is a protein that is recruited to membranes by phosphatidylinositol 3-phosphate (PI3P) and Rab5-GTP, and together these molecules facilitate early endosome fusion. From there MHC I can be either routed to late endosomes for degradation [95] or recycled back to plasma membrane [93, 95, 96]. Recycling of MHC I back to the plasma membrane requires the activity of Arf6. Thus, the possible involvement of β2M in infectious entry of many picornaviruses suggests that such viruses may also use Arf6-mediated entry pathway, and therefore Arf6 may be a common entry pathway for many picornaviruses.

4.3. Human Parechovirus 1. Currently, there are 16 human parechovirus types in the genus *Parechovirus* [3]. Parechovirus infections are common during the first years of life and often asymptomatic or related to mild gastroenteritis and respiratory infections [6, 97–100]. However, HPeV-1 and HPeV-3 may cause severe illnesses such as infections of the central nervous system, generalized infections of neonates and myocarditis [3, 97, 101]. HPeV-1, -2, -4, -5 and -6 possess an RGD motif near the C-terminus of VP1 that is known to facilitate binding of cellular ligands to integrins (Table 1) [33, 102]. HPeV-1 has been shown to interact with integrin $\alpha V\beta 1$, $\alpha V\beta 3$, and $\alpha V\beta 6$ [39, 103–105]. In contrast to CV-A9, RGD motif is essential for viability of HPeV-1; mutation in the RGD motif resulted in noninfectious phenotype, and only viruses in which the RGD sequence had been restored by reversion recovered after cell passage [106].

Although there are similarities in receptor use *in vitro* between CV-A9 and HPeV-1, the viruses seem to use different endocytic pathways. Both viruses bind to integrins $\alpha V\beta 3$ and $\alpha V\beta 6$ *in vitro*, but possess higher motif-to-motif affinity to integrin $\alpha V\beta 6$ than to $\alpha V\beta 3$. Cryo-EM studies have shown that $\alpha V\beta 6$ binds with high affinity to surface of HPeV-1 via RGD loop located in the C terminus of VP1 [39]. In contrast, β2M, which has been suggested to play role in E-1 and CV-A9 infections [72, 84, 94], may not be significant in HPeV-1 infection [105]. Despite the receptor use CV-A9 and HPeV-1 seem to be endocytosed in different manner. There is a single publication on HPeV-1 endocytosis (or endocytosis of any parechovirus), and this paper suggests that HPeV-1 uses clathrin-mediated endocytic pathway [105]. Following internalization, HPeV-1 enters early endosomes after which the virus is found in late endosomes [105]. The recent

findings from our laboratory indicate that HPeV-1 binds preferably integrin β1 and that β2M participates in the entry process (P. Merilahti and P. Susi, manuscript in preparation). It has been suggested that the clustering and activation of β1-integrins trigger caveolar endocytosis with the subsequent removal of β1-integrins from the plasma membrane [43, 107], which is evidently in contradiction with the suggestion that HPeV-1 is endocytosed via clathrin-mediated pathway [88]. On the other hand, MHC I (with β2M) has been linked to internalization of β1-integrins, but previously not shown to be involved in HPeV-1 infection [88]. It has also been demonstrated that Arf6 regulates endocytosis of β1 [108], but this has not been demonstrated for parechoviruses. Thus, further studies are needed to elucidate the role of integrin β1, involvement of β2M, and Arf6-pathway in parechovirus infection.

5. Conclusions

Although there has been means to cultivate picornaviruses since the discovery of cell culture methods in the 1950s and despite the economic importance of picornaviruses, there are still no drugs in clinical use against any picornavirus type. This is partially because of the large number of picornavirus types that infect humans, the complex nature of the infection process, and the lack of knowledge of receptor tropism and mechanisms of endocytosis. Experiments have been conducted with three pathogenic picornavirus types (CV-A9, E-9, and HPeV-1) that possess RGD motif for integrin binding. In addition, there are other human picornaviruses that possess RGD motif and may therefore use integrin(s) and cellular receptor(s) (Table 1). Yet, another integrin-binding human picornavirus, echovirus 1 (E-1), is exceptional in binding the RGD-independent integrin $\alpha 2\beta 1$. Recent studies on these viruses have shown surprisingly that whereas CV-A9 uses novel HSPA5 receptor and Arf6- and dynamin-dependent entry route in infection, cellular entry of E-1 alleviates the route of the natural ligand in integrin β1; E-1 binds inactive receptor form, and internalization is dependent on clustering and not on integrin-mediated signals. These are examples of assignment of picornaviruses to novel entry mechanisms that have not previously been described for viruses, and which differ from cellular endocytosis. Since most of the cellular studies on receptor binding and endocytosis have been conducted in cancer cell models, it remains to be determined what the relevance of detected receptor interactions and mechanisms of endocytosis in native human cells and tissues, and in model organisms, is. The future issues in such studies include the mechanism of endocytosis between viruses and natural ligands that bind the same receptor, the possible central role of β2M, the possible involvement of Arf6 pathway, and the role of novel receptors and mediators such as HSPA5 in picornavirus infection. Studies on mechanisms and mediators of endocytosis and comparisons to other picornaviruses are likely to be useful in designing receptor-targeted or intracellular drugs with broad specificities. Large-scale genomic screens and clustering analysis may also reveal common features and nominators that may be useful in drug discovery against picornaviruses in general [109]. Existing therapeutic agents against integrins may also prove to be useful as antivirals against integrin-binding human picornaviruses [110].

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Productive Entry Pathways of Human Rhinoviruses

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Currently, complete or partial genome sequences of more than 150 human rhinovirus (HRV) isolates are known. Twelve species A use members of the low-density lipoprotein receptor family for cell entry, whereas the remaining HRV-A and all HRV-B bind ICAM-1. HRV-Cs exploit an unknown receptor. At least all A and B type viruses depend on receptor-mediated endocytosis for infection. In HeLa cells, they are internalized mainly by a clathrin- and dynamin-dependent mechanism. Upon uptake into acidic compartments, the icosahedral HRV capsid expands by ~4% and holes open at the 2-fold axes, close to the pseudo-3-fold axes and at the base of the star-shaped dome protruding at the vertices. RNA-protein interactions are broken and new ones are established, the small internal myristoylated capsid protein VP4 is expelled, and amphipathic N-terminal sequences of VP1 become exposed. The now hydrophobic subviral particle attaches to the inner surface of endosomes and transfers its genomic (+) ssRNA into the cytosol. The RNA leaves the virus starting with the poly(A) tail at its 3′-end and passes through a membrane pore contiguous with one of the holes in the capsid wall. Alternatively, the endosome is disrupted and the RNA freely diffuses into the cytoplasm.

1. Introduction

Human rhinoviruses (HRVs) are icosahedral (30 nm in diameter) and nonenveloped with a (+) ssRNA genome of ~7100 bases. Belonging to the family Picornaviridae, genus *Enterovirus*, they are composed of 60 copies each of four capsid proteins, VP1 to VP4. In 1987, HRVs from clinical samples were serotyped into 100 strains [1]. Recently, complete genome sequences of all known HRVs were determined. Phylogenetic analyses grouped them into 3 species; 74 HRV-A, 25 HRV-B, and 6 HRV-C [2]. Since then, many more rhinoviruses (mostly of type C) were identified in clinical specimens [3–5]. Independent from this classification, HRV-A and HRV-B are divided into two groups based upon the receptors exploited for host cell attachment; the minor receptor group, including the so far identified 12 HRV-A, bind low-density lipoprotein receptor (LDLR), very-LDLR (VLDLR), and LDLR-related protein 1 (LRP1) [6–9], while the remaining HRVA and HRV-B (constituting the majority, that is, the major group) use intercellular adhesion molecule 1 (ICAM-1) for cell entry [10]. Some major group HRVs (HRV8, 54, and 89) can also use heparan sulfate proteoglycans (HSPG) as an additional receptor [7, 11, 12] either as wild type (wt) or after adaptation to grow in cells lacking ICAM-1. This is achieved by numerous cycles alternating between blind passages and boosting in permissive cells [13, 14]. The receptor(s) for HRV-Cs is unknown [15].

Species A and B viruses are the cause of more than 50% of all mild infections of the upper respiratory tract known as the common cold [16]. The typical symptoms are inflammatory reactions of the nasal epithelium with the release of kinins, leukotrienes, histamine, interleukin 1 (IL-1), IL-6, IL-8, TNF-α, and RANTES [17]. HRV infections are usually benign and self-limiting, but recurrent, and therefore generate enormous economic costs. In 2001, the socioeconomic burden for noninfluenza virus-related respiratory infections due to expenses for medication and working days lost amounted to $40 billion in the USA alone [18, 19]. Since 1957 [20] evidence has been accumulating

that HRVs are associated with asthma and wheeze by also infecting the lower airways. It is now well acknowledged that HRVs are involved in the exacerbations of asthma, cystic fibrosis, chronic obstructive pulmonary disease, pneumonia, sinusitis, otitis media, and wheezing of infants [21]. In addition to the costs detailed above, direct and indirect costs from such complications in asthmatics amount to $60 billion per year in the USA [22]. Global spending for respiratory infections can be estimated to be in the trillions of US dollars per year [18].

The recently discovered HRV-Cs appear to give rise to more severe respiratory tract illness especially in pediatric patients. HRV-C infections, in addition to symptoms of the common cold, cause pharyngitis, croup, otitis media, bronchiolitis, or pneumonia. This species must have circulated in the population for at least 10 years, but probably much longer, as they escaped detection because of being refractive to propagation in tissue culture [15]. The establishment of highly sensitive PCR methods now enables detection and strain typing within hours from clinical samples [3]. In hospitalized children HRV-Cs were also found in plasma, pericardial fluid, and stool samples [4, 23, 24] and the quite high concentrations question whether their replication is definitely limited to the respiratory system. Interestingly, the novel technologies detected HRV-As and HRV-Bs in fecal specimens as well. It is thus possible that all HRVs are not exclusively transmitted by the nasal/oral route but exploit a fecal-oral pathway as well. The viremia observed (preferentially) in HRV-C infections may be indicative for a distinct pathogenicity as compared to A and B viruses [16, 23].

At the time of writing, 148 HRV types had been found circulating in the human population [4]. Since recovery from infection with one serotype does not protect against reinfection with another serotype, vaccination appears difficult although common antigens have been identified [25–28]. A mixture of recombinant capsid proteins might thus be worthwhile to consider as a possible vaccine [28, 29]. Nevertheless, so far other means of disease prevention are believed to be more effective [30]. These include antiviral agents inhibiting either uncoating (by binding into a hydrophobic pocket within the capsid) or replication (by targeting virally encoded enzymes, such as the proteases). Such drugs are highly desirable for people suffering conditions like asthma where infection with a rhinovirus might heavily aggravate the symptoms. However, so far none of the initially promising compounds has reached clinical application.

2. Overview of the HRV Life Cycle

HRV infection typically involves the following sequence of events: (i) virus binding to the respective cognate receptors at the plasma membrane, (ii) entry into the cell by receptor-mediated endocytosis, (iii) transition from the native virus to a hydrophobic subviral particle, (iv) release of the viral RNA (uncoating), (v) RNA penetration into the cytoplasm, (vi) synthesis of viral proteins, (vii) RNA replication, and (viii) assembly and release of new, infectious virions.

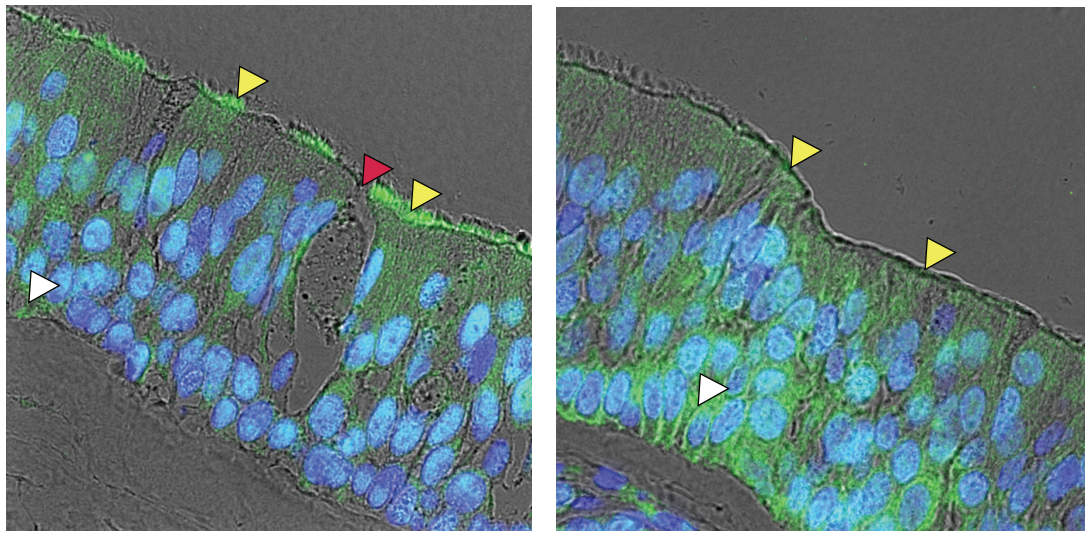
Concomitant with HRV cell entry structural changes of the viral capsid occur that ultimately result in release of the genomic RNA. It is believed that native HRVs first lose the innermost capsid protein VP4 resulting in the generation of subviral A-particles [31]; these are further converted to (empty) B-particles after the release of the RNA. This view is supported by the finding that A-particles of the related poliovirus are infective, although at a substantially reduced rate [32]. Native virions and subviral particles can be separated by ultracentrifugation; native virions sediment at 150S, A-particles sediment at 135S, and empty B-particles have a sedimentation constant of 80S [33].

Upon arrival of the viral genome in the cytosol the RNA is translated into a polyprotein that is autocatalytically and cotranslationally cleaved by the viral proteinases $2A^{pro}$, $3C^{pro}$, and its precursor $3CD^{pro}$, into structural proteins VP1, VP0, VP3, and the nonstructural proteins required for virus replication [34]. These include the RNA-dependent RNA polymerase $3D^{pol}$ as well as the precursor proteins (3CD, 2AB) that induce the formation of membrane vesicles derived from intracellular membrane-bounded compartments acting as scaffold for the RNA polymerase. Upon virus assembly maturation cleavage of VP0 into VP2 and VP4 occurs by an unknown protease [35].

3. HRV Receptors and Their Virus Binding Properties

In the following we will concentrate on the receptors for HRV-A and HRV-B since HRV-C receptors have not been identified. The physiological function and cell-type-specific expression of ICAM-1 and LDL-receptors are different. ICAM-1, a type 1 transmembrane protein, is predominantly involved in cell-cell adhesion in endothelial cells and in immune reactions by binding to the integrins lymphocyte function antigen 1 (LFA1 i.e., CD11a/CD18) and macrophage 1 antigen (Mac1, i.e., CD11b/CD18) [36]. Its extracellular part is composed of five typical immunoglobulin-like domains [37] and its short cytoplasmic tail has no known clathrin-coated pit localization signals. In cryo-EM 3D-reconstructions of complexes between soluble recombinant fragments of ICAM-1 and HRV3, HRV14, and HRV16, its first domain is seen to contact the virus inside the canyon, a cleft encircling the dome at the vertex, whereas the other domains protrude from the surface [38–41].

As will be detailed below, the structural alterations of the viral capsid of major group viruses during uncoating are catalyzed by ICAM-1 in a pH- [42] and temperature-dependent manner [43, 44]. This is similar to poliovirus where receptor binding leads to uncoating [45]; however, the structural changes of poliovirus are independent of low pH [46–49]. In contrast to ICAM-1 and the poliovirus receptor, members of the LDLR family only function in ligand internalization but do not catalyze uncoating. They transduce signals and undertake multiple rounds of recycling to the plasma membrane and reinternalization [50]. Their ligand-binding domains are composed of different numbers of ligand-binding (type A) repeats (LDLR has 7, VLDLR has 8, and LRP1 has 31). In LDLR, they are at the N-terminus and



(a) (b)

Figure 1: Receptors for minor group viruses are expressed in nasal epithelial cells. Paraffin-embedded nasal tissue sections were prepared, rehydrated, and subsequently incubated with anti-LDLR [60] or anti-LRP1 [70] antibodies, followed by the respective fluorophore-labeled antichicken or antirabbit Alexa-488-IgG. Nuclei were stained with Hoechst dye. Pictures shown are overlays of immunofluorescence and phase contrast images. LDLR (a) and LRP1 (b) are present at the apical (yellow arrow heads) surface in ciliated as well as in basal cells (white arrow heads). A mucous secreting cell is indicated by a red arrow head.

followed by three regions with similarity to the epidermal growth factor precursor (EGF-domain) containing YWTD motives forming a 6-bladed β-propeller, a domain with O-glycosylation proximal to the membrane, a transmembrane domain, and a cytoplasmic tail with an NPXY internalization motive [51, 52]. The other members of the LDLR family exhibit similar domain arrangements [53, 54]. In tissue culture cells LDLR and LRP1 mediate productive entry of minor group viruses [6]. It is likely that this also holds true for VLDLR as its downregulation inhibits infection with minor group HRVs [55]. As demonstrated for HRV2 and a recombinant fragment of VLDLR, the ligand binding repeats attachment to the top of the star-like mesa at the vertex at the five-fold axis of symmetry in a multimodular manner; thus, the binding site is distinct from that of ICAM-1 [56–59]. As mentioned above, minor group virus uncoating is receptor independent and might even be inhibited to some extent by the bound receptor; presumably, it prevents movements of the five copies of VP1 that occurs during conversion to the subviral particle [60, 61].

HRVs replicate in (polarized) epithelial cells in the upper and lower airways [62–64]. As known for HRV-A and -B viruses, only a few ciliated cells become infected and this without obvious cytopathic effect. This is in agreement with the receptor for major group viruses, ICAM-1, being expressed in only 1% of these cells in nasal tissue in the absence of inflammation [65, 66]. The very low level of ICAM-1 expression in highly differentiated ciliated epithelial cells, as compared to undifferentiated basal cells, appears to limit infection [67, 68]. No such *in vivo* data are available for minor group HRVs. Immunostaining for LDLR and LRP1 of normal nasal tissue revealed the presence of the receptors at the apical surface in ciliated as well as in basal cells. As shown in Figure 1, receptor expression between individual ciliated cells varies considerably. Neither the expression of ICAM-1 nor of LDLR/LRP1 in the nasal epithelium has been quantified *in situ*. The polarity of ICAM-1 expression is also unknown. Receptor expression obviously determines the amount of virus that can bind and enter the cells. However, the presence of a suitable receptor is not sufficient for productive infection, as subsequent events such as entry, uncoating, RNA penetration into the cytoplasm, and replication must also function properly in a given cell [69].

4. HRV Structure and Capsid Alterations during Uncoating

As all picornaviruses, HRVs possess $T = 1$, $P = 3$ icosahedral symmetry with 60 copies of each of the capsid proteins VP1, 2, 3, and 4. Except from the small internal myristoylated VP4, they fold similarly into a beta-barrel whose eight antiparallel beta-sheets are connected with long (external) and short (internal) loops. The former are exposed and make up the antigenic sites, targets for type-specific antibodies [71–76]. The inner capsid wall is stabilized by an intricate network built by interacting residues of the N-terminal extensions of VP1 and VP2 under contribution of the backbone of VP3; residues of both VP1 and VP2 interact with the RNA [77].

Concomitant with HRV cell entry the viral capsid suffers structural alterations. Induced by interacting with ICAM-1 (major group HRVs, [44, 78, 79]) or exclusively triggered by the low pH (minor group HRVs) the virus loses the pocket factor, presumably a fatty acid residing in a void within VP1, and expands by about 4% [80, 81]. In major group viruses, depending on the serotype, the low pH may

aid the "catalytic" function of ICAM-1 [42]. For HRV2, it was recently shown that on expulsion of the pocket factor, the empty space allows for Met^{213} of VP1 to move in. This results in a substantial part of the chain pivoting over this site [80]. The final result is a loosing of intra- and intersubunit interactions and the opening of three types of holes [77]; the largest ones at the twofold axes and smaller ones at the pseudo three-fold axes and at the base of the star-shaped domes at the vertices. The small innermost myristoylated capsid protein VP4 escapes (its exit site is unknown but the holes are big enough for an unfolded protein to pass) and N-terminal sequences of VP1 become exposed most probably on exiting through the pore close to the pseudo-threefold axes [77]. A similar exit point has been proposed earlier for HRV3 [38] and poliovirus [82–84]. Due to the amphipathic nature of the N-terminal VP1 sequences these A-particles then attach to endosomal membranes and release the RNA. Native virus is thereby converted into B-particles (i.e., empty capsids).

Recent cryo-EM data demonstrated that the RNA is much more structured than previously thought; in addition to the well-known contacts with the conserved Trp^{38} of VP2 ([85] and references therein) it also interacts with residues of VP1. These latter are part of an interaction network contributed by the N-terminal extensions of VP1 and VP2. Comparison of the 3D X-ray structures of native HRV2 and its empty capsid and a model of the 135S-particle at close to atomic resolution reveals that this network is broken in the subviral particles [77]. Since a number of acid-sensitive residues are nearby it is likely that their protonation weakens this network letting go the RNA to escape through one of the holes. In A-particles the RNA-protein contacts have changed with respect to the native virion; those at the 2-fold axes are maintained but new ones are being established with N-terminal residues of the VP3 ß-cylinder that have become accessible after VP4 has escaped.

5. HRV Entry Pathways and Intracellular Trafficking

5.1. Entry into Tissue Culture Cells. Early on, the low pH sensitivity of HRVs suggested cell entry by endocytosis and uncoating in endosomal compartments [31, 33, 86]. Endocytosis, the uptake of extracellular material within membrane-bound vesicles, has first been described by Metschnikoff about 130 years ago [87]. It starts by binding of ligands to specific receptors, concentration of these complexes in specialized domains at the plasma membrane (e.g., coated pits, caveolae, and lipid rafts) followed by membrane invagination and pinching off of primary endocytic vesicles. Viruses have proven to be valuable tools for studying the mechanisms of primary endocytic vesicle formation [88]. So far, clathrin-mediated endocytosis is best characterized [89]. It depends on particular sequence motives in the cytoplasmic tail of the transmembrane receptors for clathrin-coated pit formation [90]. Caveolae-dependent as well as clathrin- and caveolin-independent pathways are less defined. In addition to specific coat proteins (clathrin, caveolin, flotillin) and accessory molecules (adaptor proteins), the GTPase dynamin plays an essential role in the constriction/fission process during clathrin-, caveolin-, and lipid-raft-mediated uptake [91]. Based on the distinct requirements for clathrin, caveolin, dynamin, cholesterol, and various other accessory molecules, 10 different endocytic pathways have been differentiated so far [88]. Irrespective of the uptake mechanism, internalized receptors and ligands are first delivered to early (sorting) endosomes [92–95]. From early endosomes, internalized material can then follow different intracellular routes [95, 96]: (i) transport to lysosomes, resulting in degradation of ligands and certain receptors, (ii) recycling to the cell surface, and (iii) in polarized cells, transport from one plasma membrane domain to the opposite plasma membrane domain (transcytosis).

A main feature of endosomes is their ability to acidify their interior by a vacuolar proton ATPase (V-ATPase) [97–99]. Due to the concerted action of the V-ATPase, Na^+/K^+-ATPase, transporters, and ion channels, distinct pH values are established in endocytic subcompartments that play an important role in trafficking of macromolecules through endocytic pathways, in ligand degradation, and inactivation of internalized pathogens [100, 101]. Although endocytic-coated vesicles may not be acidic [102], the mildly acidic pH in early endosomes causes the dissociation of many ligands from their receptors [103] allowing for receptor recycling; a small fraction of internalized fluid containing the released ligands is routed through late endosomes to lysosomes for rapid degradation (Figure 2). The formation of "nascent" late endosomes may involve budding and fission events from early endosomes resulting in "endosomal carrier vesicle" (ECV) formation [104]. In any case, nascent late endosomes undergo a sequence of maturation events until fusion with lysosomes can take place [101]. Material *en route* to lysosomes (pH 4.5–4.0) is exposed to an increasingly acidic pH during transport from early endosomes (pH 6.5–6.0) through ECV/late endosomes ($pH \leq 5.6$) [100, 105–108]. Concomitantly with the decrease in pH the internal ionic milieu of endosomes undergoes major alterations as compared to the outside environment [99]; the calcium and chloride concentration first decreases and subsequently increases, and the continuous decrease in sodium ions is paralleled by potassium ion increase. These alterations are brought about by ATPases, transporters, channels, and passive ion permeabilities in the membrane of endosomes.

Receptors (e.g., the transferrin receptor, LDLR), certain ligands (e.g., transferrin), plasma membrane proteins, and the majority of internalized fluid are recycled to the cell surface [95]. As exemplified by transferrin, recycling can occur via two pathways; from early endosomes with $t_{1/2} \approx 2$ min (fast) and from the perinuclear recycling compartment (PNRC) with $t_{1/2} \approx 12$ min (slow) [109, 110]. In CHO and Hep2 cells, the pH of the PNRC is higher than that of early endosomes, whereas it is more acidic than in early endosomes in HeLa cells [95, 105].

Due to distinct mechanisms of endosomal transport to lysosomes and recycling to the plasma membrane, different drugs and dominant negative mutants, for example, of rab GTPases, may be used to arrest ligands, receptors, and

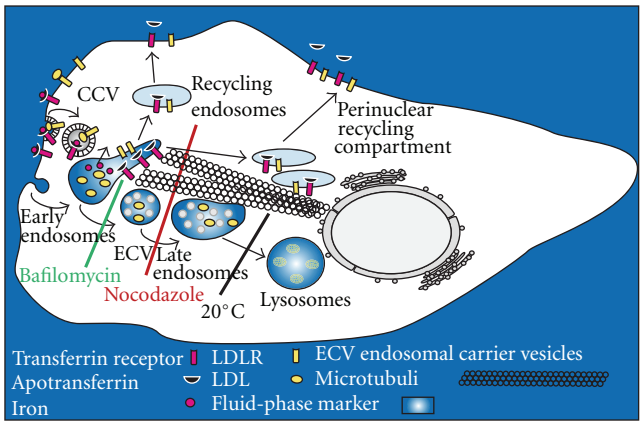


FIGURE 2: Influence of bafilomycin, nocodazole, and low temperature on endocytic pathways in HeLa cells. The recycling pathway (transferrin, LDLR) and the transport of ligands (LDL) and fluid-phase marker to lysosomes are shown. Iron-loaded transferrin binds to its receptor at the plasma membrane. The complex is internalized via clathrin-coated vesicles (CCV) and delivered into early endosomes within 2–5 minutes, where the iron is released and transferred into the cytoplasm. Apotransferrin remains bound to the receptor and recycles via a fast and a slow pathway. At the plasma membrane, apotransferrin is released at the neutral pH. Similarly, internalized LDL is released from its receptor in early compartments allowing for LDLR to return to the plasma membrane via the same pathways as apotransferrin. Although a major portion of fluid-phase marker (e.g., dextran) is recycled, the remaining fluid and released ligands (LDL) are delivered from early endosomes (within 5 min), via endosomal carrier vesicles (ECV) and late endosomes (within 15 min), to lysosomes (within 25 min). Transferrin transport to and recycling via the perinuclear recycling compartment is blocked by nocodazole, whereas bafilomycin and lowering the temperature to 20°C are without effect. In contrast, bafilomycin arrests fluid-phase markers in early endosomes by preventing budding of ECV, whereas nocodazole leads to accumulation of cargo in ECV. Finally, incubation at 20°C prevents delivery of markers from late endosomes to lysosomes. For further details and endosomal pH determination see [105, 108]. Adapted from Fuchs and Blaas [112].

fluid in specific endosomal subcompartments (see Figure 2). Furthermore, when ligands destined to lysosomes are taken up at reduced temperature (e.g., at 20°C), internalization takes place (albeit at reduced rate) and sorting does occur but fusion of late endosomes and lysosomes is prevented [111]. In contrast, the kinetics of transferrin endocytosis and recycling is unaffected at 20°C [105]. Consequently, these treatments are valuable tools for investigating whether viruses follow a recycling or degradative pathway and to identify the compartment where virus penetration/uncoating takes place.

One particular virus can use multiple entry pathways [113–115], but not all may result in delivery to the compartment where the internal milieu allows for the structural alterations and genome release (i.e., for productive uncoating) leading to infection of the host cell. Why is it important to define the virus entry route and the compartment of productive uncoating and genome penetration? Since distinct cellular factors are necessary for virus internalization and intracellular routing these molecules may represent potential drug targets for antivirals. For example, Urs Greber's group recently demonstrated that niclosamide, an antihelminthic drug approved by the FDA since a long time, prevents infection by various HRVs via neutralizing acidic endosomes [116]. Another example is HIV; this enveloped virus penetrates into the cytoplasm by fusion with the plasma membrane but it can be routed into an unproductive pathway by enhancing its endocytosis [117, 118]. Redirection from a productive to an unproductive endocytic compartment was also shown for coxsackievirus B3, another picornavirus [119].

Combining small molecule inhibitors, dominant-negative mutants, RNAi, immunofluorescence microscopy, FISH, and subcellular fractionation with infectivity assays, we have been studying the productive entry route(s) of HRVs into tissue culture cells [12, 108, 120–125]. Because of the high particle to infectious particle ratio (between 24 : 1 and 240 : 1; [126] or even much higher [127]) determining infectious virus was and is of particular importance in such investigations.

The entry mechanism of major group HRVs appears to by cell-type specific. In HeLa cells (Figure 3(a)), HRV14 uptake occurs by a dynamin- and presumably clathrin-dependent route [128, 129], whereas endocytosis in ICAM-1 overexpressing rhabdomyosarcoma cells is independent of clathrin, caveolin, flotillin, and lipid rafts [123]. HRV14 endocytosis and productive uncoating in these cells were partially inhibited by blocking dynamin function with dynasore and by disrupting the actin cytoskeleton. Conversely, the Na^+/H^+ exchange inhibitor amiloride prevented uptake and uncoating indicative for macropinocytosis as infectious entry route [123]. Some major group viruses (HRV8, HRV54, and HRV89) either use or can be adapted to use HSPG as an alternative receptor for cell binding in addition to ICAM-1 [11–13]. Entry and infection of the HSPG-binding variant of HRV8 (HRV8v) in rhabdomyosarcoma cells devoid of ICAM-1 were very similar to entry and infection of HRV14 in these cells [12]. Our recent studies on the productive entry route of HRV89, another major group virus, in HeLa cells are in agreement with a clathrin- and dynamin-dependent mechanism [130]. This is surprising since ICAM-1 has no known clathrin-coated pit localization signals in its cytoplasmic tail and GPI-linked ICAM-1 can mediate HRV14 internalization and infection [131]. After plasma membrane binding, HRV14-ICAM-1 complexes are delivered into mildly acidic early endosomes [124, 132]. It has not been investigated *in vivo* whether major group HRVs dissociate from ICAM-1 at low endosomal pH, as suggested from *in vitro* experiments [133]. Neither is the further trafficking of major group HRVs in HeLa cells completely clear. By using immunofluorescence microscopy, HRV14 was found in early and late endosomes when internalized at 20°C [124] but not when uptake occurred at 34°C. Under the former condition the conformational modification of the capsid catalyzed by ICAM-1 and thus infection is inhibited. These results contradict recent data by Khan et al. [123] where HRV14 exhibited some colocalization with the fluid-phase marker dextran after co-internalization at 34°C in ICAM-1 overexpressing rhabdomyosarcoma cells. Whether

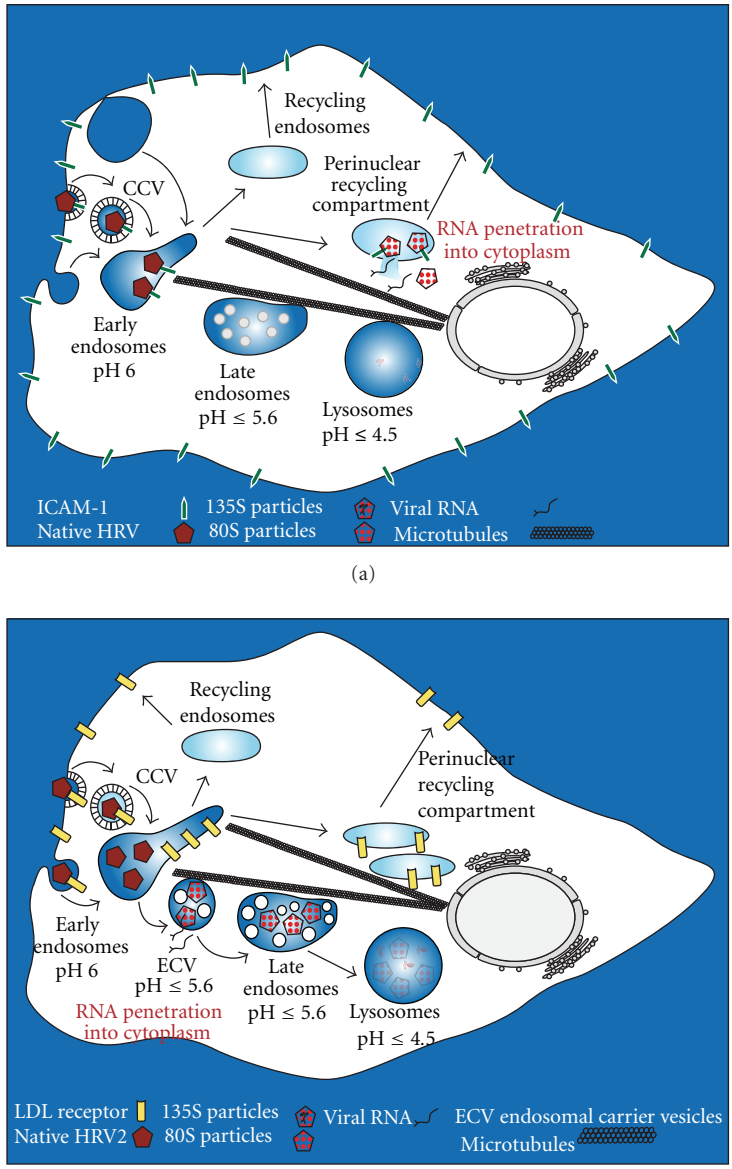


Figure 3: Entry, intracellular trafficking, and uncoating of HRVs in HeLa cells. (a) The major group virus HRV14 is internalized via clathrin-mediated endocytosis and delivered into early endosomes from where it presumably further traffics into the perinuclear recycling compartment. Structural modification of the viral capsid catalyzed by ICAM-1 is probably coupled to RNA release and rupture of the endosomal membrane. These events lead to delivery of free RNA and empty capsids into the cytoplasm. Most likely, uncoated virus and the RNA escape from the perinuclear recycling compartment. (b) HRV2 enters via clathrin-dependent and independent pathways and dissociates from its receptors at mildly acidic pH in early endosomes. Receptors are recycled and HRV2 is transferred to ECV/late endosomes where the more acidic pH (≤5.6) induces the structural modification resulting in uncoating and RNA transfer into the cytosol. Finally, residual native virus, subviral particles, and viral RNA are transported via late endosomes to lysosomes where they are degraded. Adapted from Fuchs and Blaas [112]. Note that the pathways might differ in other cell types.

this discrepancy is due to ICAM-1 overexpression or the different cell type remains to be demonstrated. As already shown by Lonberg-Holm and Korant [31] the virus is apparently not transported to lysosomes as viral RNA is not degraded after 60 min incubation at 34°C. Collectively, these data may indicate that HRV14 follows the recycling pathway, is targeted to other organelles, or disrupts the endosomes for escape (Figure 3(a)).

The so far investigated major group HRVs were found to be dependent on ICAM-1 for capsid modification; some HRVs are additionally "primed" by low endosomal pH for receptor-catalyzed uncoating [42]. Infection by these serotypes is either completely (HRV16), partially (HRV14), or not (HRV3) blocked by raising the endosomal pH with bafilomycin [42, 132]. Inability of bafilomycin to completely block infection by major group HRVs would indicate

RNA uncoating/penetration in/from early endosomes or the PNRC since this drug not only increases endosomal pH but also halts transport of cargo to late endosomes [134]. In contrast, transfer from early endosomes to the PNRC persists in the presence of bafilomycin [105, 135].

HRV2, a prototype minor group HRV, is internalized by clathrin-mediated endocytosis into early endosomes (Figure 3(b)) [125]. However, when this pathway is blocked by cytosol acidification or overexpression of nonfunctional dynamin, HRV2 enters via a clathrin- and dynamin-independent pathways [122, 136]. Similar to the natural ligand LDL the virus dissociates from its receptors in early endosomes. Obviously, the specific internal milieu in these compartments (low calcium) facilitates dissociation by destabilizing the conformation of the ligand-binding repeats. This is supported by the finding that little LDL and HRV2 are released from the receptors at the plasma membrane at pH 6.5–6.0 [52, 60, 137]. Furthermore, LDLR and HRV2 release depend on intramolecular competition with the beta-propeller domain of the receptor [60]. LDLR is recycled to the plasma membrane [60] and the virus is targeted via typical LAMP-positive late endosomes to lysosomes where it is rapidly degraded [31, 138]. Impeding HRV2-LDLR dissociation by deletion of the beta-propeller and the EGF-C domain of human LDLR results in degradation of the entire complex and thus in receptor downregulation [60]. In contrast to major group HRVs and their receptor ICAM-1, uncoating and membrane penetration can take place in the absence of LDLR/LRP and this process is solely dependent on pH $\leq$ 5.6 *in vitro* and *in vivo* [139, 140]. In accordance with this pH requirement, HRV2 has been found to release its RNA in ECV/late endosomes *in vivo* [124, 138].

5.2. Entry into Airway Epithelial Cells. Although HRV replication in HeLa cells and in primary human bronchial epithelial cells is comparable [141], HeLa cells are not a valid model for the airway epithelium. The respiratory epithelium is built from different cell types; predominantly ciliated columnar (epithelial) cells and mucous-secreting goblet cells (Figure 1). Both are polarized with their apical and basolateral plasma membrane separated by tight junctions. The basal cells are small and rounded and are in contact with the basal lamina; they can differentiate into the other cell types [142]. For studying the mechanisms of virus replication outside the human body either organ cultures of biopsy material [143] or cultures of primary nasal, tracheal cells, bronchial epithelium [144], or immortalized airway cell lines (Calu3, 16HBE) [145] were used. Since no comparative studies on the productive entry pathways of HRVs in all these systems have been carried out, it is unknown which cell line or cell type would best represent the *in vivo* situation.

The clinical symptoms of an HRV infection are the consequence of the immune response of the infected respiratory epithelium. Thus, it is apparent that HRV binding to its receptor and virus entry activate various signaling pathways leading to secretion of inflammatory mediators. The nonreceptor protein tyrosine kinase Syk has been identified as an early signaling molecule that ultimately leads to IL-8 expression [146, 147]. Upon binding of HRV16 to ICAM-1 in primary bronchial epithelial cells Syk is recruited to the plasma membrane together with ezrin that in turn can interact with filamentous actin. Both Syk and ezrin associate with clathrin in response to virus binding. These data suggest—in agreement with studies in HeLa cells—that the major group virus HRV16 enters via a pathway involving clathrin and actin. Following virus entry, Syk and ezrin appear to redistribute from the plasma membrane to endosomal compartments [148] suggesting that they might continue signaling from endosomes. Syk recruitment to ezrin also results in activation of the p85 regulatory subunit of the phosphatidylinositol (PI) 3-kinase and the Akt signaling pathway [147, 149]. Another target activated by binding of the major group virus HRV39 to ICAM-1 that is also involved in regulation of IL-8 expression has been shown to be Src [149, 150]. Src functions as an upstream regulator of p110β catalytic subunit of PI 3-kinase (that in turn forms a complex with p85 PI 3-kinase) and of Akt. Furthermore, HRV39 internalization and/or intracellular trafficking appear to depend on PI 3-kinase activation [149].

In contradiction to the observations described by Lau et al. [148], Dreschers and coworkers noticed the induction of ceramide-enriched membrane domains by major group and minor group viruses in nasal mucosa, isolated nasal epithelial cells, HeLa cells, and fibroblasts [151, 152]. Such domains may be important for p38-MAPkinase activation in response to HRV14 infection [153] as well as for HRV39-induced Src signaling in airway cells [150]. Remarkably, viral replication is not required for activation of p38-MAPkinase. Thus, most likely, receptor clustering by the multivalent virus on the one hand induces signals facilitating virus entry and/or replication and, on the other hand, signals upregulating the immune response.

Despite considerable information on induction of inflammatory mediators by minor group viruses and inhibition of infection by various drugs, essentially no data have been published on the mechanism of entry and uncoating of minor group HRVs in airway cells. *In situ*, infection by major group HRVs leads to inflammation and increased ICAM-1 expression [66]. It is thus interesting that LDLR expression was found to be upregulated upon infection with the major group virus HRV14 or the minor group virus HRV2 in primary cultures of human tracheal epithelial cells [144]. *Vice versa*, in the same cells, ICAM-1 expression was also increased after infection with HRV14 or HRV2 [154]. These effects may be explained by HRV-induced activation of nuclear factors SP1 and (NF)-κB that regulate both ICAM-1 and LDLR expressions.

6. Mechanism of RNA Uncoating

6.1. Penetration and Uncoating of Major Group Viruses. Although major group HRVs bind ICAM-1 at 4°C, the receptor-catalyzed structural modifications required for RNA release only occur at temperatures $\geq$26°C *in vitro* (shown for HRV3) [44] as well as *in vivo* (HRV14)[155]. In agreement with the inability of ICAM-1 to induce virus uncoating below 26°C, HRV14 was found in endosomes when internalized at 20°C. Although the pH in this

compartment is about 5.6 [138] this is not sufficient for HRV14 uncoating in the absence of the receptor [156]. However, when the virus was internalized at 34°C, a condition where the viral RNA is released, HRV14 was not detected in endosomal compartments. We thus concluded that it penetrates into the cytoplasm by rupturing the endosomal membrane [124]. Further evidence for this mechanism was derived from different experimental approaches; (i) by electron microscopy, free HRV14 was seen in the cytosol 30 min after entry [129]. (ii) HeLa cell endosomes were labeled with the pH-sensitive FITC and the pH-insensitive Cy5-dextran. FACS and single-organelle flow analysis (SOFA) [120] demonstrated an increase in the mean pH from 6.0 (control, in the absence of virus) to 7.0 upon co-internalization of these fluid-phase markers with HRV14 (see Figure 3 in [112]). This correlated with a reduction in the number of the labeled endosomes by 23% indicating that the fluorescent markers had been released into the pH neutral cytoplasm. For comparison, adenovirus was analyzed in parallel. This virus is known to penetrate into the cytosol by very efficient endosome lysis, reducing the number of fluid-phase marker-labeled endosomes by 37%. At least in HeLa cells, uncoating and subsequent infection of HRV14 can also take place when the endosomal pH is neutralized by bafilomycin [132]. This drug, in addition to inhibition of V-ATPases, arrests markers en route to lysosomes in early endosomes [134]. In its presence HRV14 was not detected in isolated endosomes suggesting that the virus penetrates into the cytosol by rupture of early endosomes. Since the recycling pathway is not affected by elevating the endosomal pH it might be also considered that the virus penetrates from and ruptures the PNRC [105, 135]. Nevertheless, as long as the RNA has not been traced on its way from within the intact capsid into the cytosol, the localization of the process will remain indirect and lacking definite proof.

6.2. Uncoating and RNA Penetration of HRV2. In contrast to HRV14, HRV2 was localized in ECV/late endosomes in intact HeLa cells by immunofluorescence microscopy as well as by subcellular fractionation of isolated endosomes [124, 157]. Various experimental setups support a mechanism where the RNA is transferred from these compartments into the cytoplasm through a pore in the membrane [120, 121, 124]. As recently shown, under conditions of productive uncoating, HRV2 induces ion permeable channels presumably lined by viral proteins (see Figure 6 in [112]). Most likely, the RNA travels through these pores into the cytosol. RNA transfer is stimulated by a *trans*-negative membrane potential (endosome interior positive) as compared to inside positive potential but is unaffected by the pH gradient between endosomes and cytoplasm [158].

Taken together, at present, data on rhinoviruses and poliovirus from different laboratories support a model in that VP4 and the amphipathic N-terminal extensions of VP1 insert into the membrane of late endosomes to form an ion-conducting pore. This channel would be exploited by the RNA to pass. The previous, quite suggestive model positioned the exit site of the RNA at a fivefold axis; this places five receptor molecules and five copies of the VP1 N-terminal extensions upright onto the membrane. However, according to the present model RNA exit occurs at a twofold axis leaving us with the question of how this opening in the viral shell can be positioned on the membrane (by receptors and/or the amphipathic extensions of the capsid proteins of the subviral particle) in a way as to form a contiguous channel.

RNA exit from poliovirus inside the cell has been localized to endosomal compartments by life cell microscopy [159] and membrane penetration of the viral genome has been demonstrated in intact liposomes on acidification of bound HRV2 [140]. Nevertheless, direct visualization of the RNA passing through this putative channel is lacking.

In contrast to a number of older textbook illustrations, at least *in vitro*, the RNA leaves the virion with its 3′-end first and not with the 5′-end carrying the peptide VPg [160]. Since the 5′-end is being synthesized first, encapsidation is likely to start with this end and might terminate with the poly-(A) tail left close to the location where a hole is going to open at one of the 2-fold axes when the subviral particle forms [161, 162].

7. Future Perspectives

Although considerable progress has been made in unraveling the entry mechanisms of rhinoviruses into tissue culture cells, amazingly little is known on the entry route leading to productive infection of the airway epithelium. Identification of receptors for HRV-C type viruses, characterization of their entry pathways, and comparison with A and B types will shed light on the distinct pathology of infection caused by this virus species. A systems biology approach in combination with new high-throughput technologies may lead to identification of cellular host factors essential for HRV entry, trafficking, uncoating, signaling, and replication and thus point to novel drug targets [163, 164]. Structural studies using electron microscopy and X-ray crystallography of viruses and virus-(receptor)-liposome complexes in combination with novel technologies have shed light on the capsid modifications occurring during uncoating and the mode of RNA exit. Such techniques need now to be utilized to study the *in vivo* situation, that is, to characterize the molecular mechanisms of virus-endosome interaction and to visualize the RNA during transit from the protective viral shell through a membrane into the cytosol.

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Review Article

Features of Human Herpesvirus-6A and -6B Entry

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Human herpesvirus-6 (HHV-6) is a T lymphotropic herpesvirus belonging to the *Betaherpesvirinae* subfamily. HHV-6 was long classified into variants A and B (HHV-6A and HHV-6B); however, recently, HHV-6A and HHV-6B were reclassified as different species. The process of herpesvirus entry into target cells is complicated, and in the case of HHV-6A and HHV-6B, the detailed mechanism remains to be elucidated, although both viruses are known to enter cells via endocytosis. In this paper, (1) findings about the cellular receptor and its ligand for HHV-6A and HHV-6B are summarized, and (2) a schematic model of HHV-6A's replication cycle, including its entry, is presented. In addition, (3) reports showing the importance of lipids in both the HHV-6A envelope and target-cell membrane for viral entry are reviewed, and (4) glycoproteins involved in cell fusion are discussed.

1. Introduction

The *herpesviridae* are a family of double-stranded enveloped DNA viruses. Their entry into host cells proceeds as follows. First, the virus binds to its target cell through a specific receptor. Second, herpesviruses enter cells via two different pathways: (a) direct fusion of the viral envelope with the target-cell plasma membrane or (b) endocytosis followed by fusion between the viral and cellular membranes in the endosomal compartment [1].

Human herpesvirus-6 (HHV-6) was initially isolated from the peripheral blood of patients with lymphoproliferative disorders, in 1986 [2]. It belongs to the *Betaherpesvirinae* subfamily, along with human cytomegalovirus (HCMV) and Human herpesvirus-7 (HHV-7), and is a member of the genus *Roseolovirus*, along with HHV-7. HHV-6 was originally classified into variants A and B (HHV-6A and HHV-6B), based on differences in genetic, antigenic, and growth characteristics [3–5]. However, recently, HHV-6A and HHV-6B were reclassified into different species (Virus Taxonomy List 2011). The homology of entire genome sequence between both is nearly 90% [6–8]. Primary infection of HHV-6B causes exanthem subitum [9], and HHV-6A has been reported to be involved in several diseases, including encephalitis [10], hepatitis [11], glioma [12], and multiple sclerosis [13].

However, the detailed replication cycle of HHV-6A and HHV-6B after entering the cell remains to be elucidated. For some of the steps, different groups have reported conflicting results.

Regarding the ligand and receptor for HHV-6A and HHV-6B, Santoro et al. reported that the cellular receptor for both viruses is CD46 [14]. Our group showed that the glycoprotein gH/gL/gQ1/gQ2 complex [15] is the ligand for HHV-6A [16], and that its receptor is CD46 [17, 18]. However, in the case of HHV-6B (strain HST, an isolate from an infant with exanthem subitum), we reported that, of two complexes found in this virus, gH/gL/gQ and gH/gL/gO neither binds to CD46 [19]. This discrepancy might be due to the difference in HHV-6B strain (Santoro et al. used strains Z29 and PL1, while we used HST) or some other reason.

In this paper, (1) previous reports about the cellular receptor and its ligand for HHV-6A and HHV-6B are summarized, and (2) findings about the entry of HHV-6A and HHV-6B into host cells are reviewed, and a schematic model of HHV-6A's replication cycle is presented. In addition, (3) reports showing the importance of lipids in both the HHV-6A envelope and target-cell membrane for viral entry are

reviewed. Finally, (4) glycoproteins that have been shown to play a critical role in cell fusion (glycoprotein H, glycoprotein B) are discussed briefly.

2. The Cellular Receptor and Its Ligand for HHV-6A and HHV-6B

2.1. HHV-6A. As described above, Santoro et al. reported that HHV-6A and HHV-6B use the human complement regulator CD46 as a cellular receptor [14], and our group showed that the gH/gL/gQ1/gQ2 complex of HHV-6A binds to CD46 [17]. Santoro et al. identified gH as the CD46-binding component of HHV-6A. They showed that (i) an anti-CD46 antibody immunoprecipitated gH in HHV-6A-(GS-strain-) infected cells, (ii) the anti-CD46 antibody did not immunoprecipitate gH from gH-depleted HHV-6A-infected cells, although it did immunoprecipitate gH from gp82-105- or gB-depleted HHV-6A-infected cells, and (iii) a specific anti-gH antibody immunoprecipitated CD46 in HHV-6A-infected cells [20].

Our group showed that not only an anti-gH antibody but also an anti-gQ1 antibody could immunoprecipitate CD46 from HHV-6A- (GS-strain-) infected cells [18]. Furthermore, we recently confirmed that an anti-gQ1 monoclonal antibody (Mab) immunoprecipitates CD46 from HHV-6A-(GS-strain-) infected cells (unpublished data) and showed that CD46 immunoprecipitates gH, gL, gQ1, and gQ2 from cells transfected with all four genes [17]. We also showed that the maturation of gQ1, which is indicated by its size shift from 74 kDa (gQ1-74 K) to 80 kDa (gQ1-80 K), occurs only when all four components, gH, gL, gQ1, and gQ2, are coexpressed, and that gQ1-80 K, but not gQ1-74 K, contributes to the gH/gL/gQ1/gQ2 complex and binds to CD46 [17].

CD46 is a member of the glycoprotein family called regulators of complement activation (RCA) [21, 22]. CD46 is a type I transmembrane glycoprotein of 45–67 kDa expressed on all nucleated cells [23]. CD46 contains four short consensus repeats (SCRs) in its N-terminal region, followed by a serine-threonine-proline (STP) rich domain, a small region of unknown significance, a transmembrane domain, and a cytoplasmic tail. Four distinct CD46 isoforms generated by alternative RNA splicing are expressed differentially in various cell types; these isomers have the same SCRs but different STP or cytoplasmic domains [21]. CD46 functions as a cofactor in the factor-I-mediated proteolytic cleavage of C3b and C4b; this process protects host cells from inadvertent lysis by the complement system. CD46's interactions with C3b and C4b involve several regions on SCR2, SCR3, and SCR4 [24]. CD46 was recently reported to have roles not only in innate immunity but also in adaptive immunity [23]. Furthermore, it was demonstrated that CD46 induces autophagy upon pathogen recognition [25].

CD46 is also used as an entry receptor for several human viruses and bacteria. SCR1 and SCR2 are critical domains for measles virus, SCR3 and the STP region for Neisseria gonorrhoeae, and SCR3 and SCR4 for group A streptococcus [24]. For HHV-6A, our group showed that SCR2, SCR3, and SCR4 are required for virus-mediated cell-cell fusion [26], although other groups reported that SCR2 and SCR3 are the critical determinants for CD46 binding [20] and cell fusion [27]. The reason for the discrepancy is uncertain. It is possible that the direct binding domains are in SCR2 and SCR3, while SCR4 is required only to maintain the conformation of the binding site, because Santoro et al. replaced the SCR4 of CD46 with that of DAF (decay accelerating factor), whereas we deleted SCR4. Further investigation is required to clarify this issue. The structure of extracellular portion of CD46 was recently reported [24], but that of its HHV-6A ligand, the gH/gL/gQ1/gQ2 complex, still needs to be determined.

Of the four components of the HHV-6A gH/gL/gQ1/gQ2 complex, our group reported that gQ1 and gQ2 are essential for viral infection [17, 28]. We also showed that, in addition to gH/gL/gQ1/gQ2, the HHV-6A viral envelope contains the complex gH/gL/gO, which does not bind to human CD46 [19]. The specific molecular function of HHV-6A gO and the gH/gL/gO complex remains to be elucidated. In HCMV, the gH/gL/gO complex is necessary for viral entry into human fibroblasts [29]. In addition, in EBV, the gH/gL complex associates with gp42, and this gH/gL/gp42 complex is necessary for viral entry into B cells but not into epithelial cells [30]. CMV also encodes glycoproteins that redirect cell tropism by forming complexes with gH/gL [31]. The predicted amino acid identity between the gO of HHV-6A and HHV-6B is 76.8%, which is much lower than that of other glycoproteins. Therefore, the gH/gL/gO complex may confer different biological properties in HHV-6A and HHV-6B, including cell tropism. Efforts to elucidate the function of gO and gH/gL/gO are underway.

2.2. HHV-6B. In 1999, Santoro et al. reported that CD46 is the receptor for both HHV-6A and HHV-6B [14]. They showed that (i) an anti-CD46 antibody inhibited HHV-6B (strain Z29) infection and HHV-6B-mediated cell fusion in PBMCs and (ii) the expression of CD46 in NIH3T3 cells (mouse fibroblasts) and EL4 cells (murine T lymphoblasts) caused HHV-6B- (strain-PL1-) mediated fusion and entry, respectively. In addition, Pedersen et al. reported that HHV-6B (strain PL1) causes fusion from without (FFWO) in HEK293 and SupT-1 cells [32, 33].

However, our group showed that the HHV-6B (strain HST) virion contains gH/gL/gO and gH/gL/gQ complexes, but that neither of them binds to CD46 [19], and that HHV-6B (strain HST) does not mediate FFWO in various cell types expressing human CD46, except for MT4 cells [26]. The discrepancy between these results could be attributable to several differences, including the titer of HHV-6B infection, and the HHV-6B virus strain.

Regarding the glycoprotein complex, it has been found that HHV-6B gH/gL/gQ1/gQ2 complex also plays an important role for the entry [34].

3. Replication Cycle of HHV-6A and HHV-6B

3.1. HHV-6A. As shown above, entry of herpesviruses into cells occurs in two distinct steps. In 1992, Cirone

et al. showed that HHV-6A (strain GS) enters the T-lymphoblastoid cell line, HSB-2 cells, via endocytosis and that no fusion event occurs at the plasma membrane [35]. Since then, no other reports have been shown regarding the HHV-6 entry step. For HHV-6A assembly, the envelopment-deenvelopment-reenvelopment pathway has been proposed [36, 37]. In this model, HHV-6A assembly occurs as follows. (i) The intranuclear naked capsid (around 80 nm in diameter) buds into the perinuclear cisternae, and the nucleocapsid acquires a primary envelope, which is devoid of glycoprotein. (ii) Deenvelopment of the nucleocapsid occurs in the cytoplasm, as shown by the presence of cytoplasmic naked nucleocapsids (around 140 nm in diameter). (iii) The naked nucleocapsid again acquires an envelope as well as spikes in cytoplasmic vesicles, and, finally, mature viral particles (around 185 nm in diameter) form. However, many details of this process remain to be elucidated.

Different models have been proposed for tegumentation and for the compartment in which HHV-6A reenvelopment occurs. Regarding tegument acquisition, Roffman et al. reported that HHV-6B (Z29 strain) virions in infected thymocytes acquire their tegument in tegusomes, which are spherical intranuclear compartments resulting from cytoplasmic invagination into the nucleus, because they contain ribosomes [38]. However, Torrisi et al. could not find such structures in HHV-6A strain GS-infected HSB-2 cells [36]. Later, Ahlqvist et al. suggested that tegumentation of HHV-6A can occur in the nucleus, in the tegusome or in the cytoplasm, or in either of the compartment from the analysis with U1102 infected SupT-1, lymphoblastoid cell line, and HPDA(human progenitor-derived astrocytes) [37]. The different results obtained in these reports could be due to differences in the virus (HHV-6A or HHV-6B), viral strain (HHV-6A GS or U1102), or type of cells used.

As to the reenvelopment compartment, since viral glycoproteins (gB and gH) are absent from the HHV-6A- (strain-GS-) infected HSB-2 cell plasma membrane, the plasma membrane is unlikely to be the site for reenvelopment [39, 40]. Torrisi et al. suggested that reenvelopment at annulate lamellae (AL) is required for HHV-6 to acquire an envelope with spike protein [36]. Cardinali et al. reported that HHV-6A- (strain-GS-) infection induced AL in HSB-2 cells, and proposed the AL as a putative site for oligosaccharide addition, based on the results of labeling with HPL (Helix pomatia lectin, which recognizes intermediate forms of glycoconjugates after the O-linked addition of sugar in cis-Golgi cisternae) and WGA (Wheat germ agglutinin, which binds terminally glycosylated components) [41]. However, Ahlqvist reported that no AL were found in HHV-6A- (strain-U1102-) infected astrocytes (HPDA), and that although a low percentage of U1102-infected SupT-1 cells had AL, no viral particles could be found in them. The authors suggested that the spikes of mature viral particles are acquired at cytoplasmic vesicles of unknown origin [37].

Recently, our group showed that HHV-6A (strain GS) induces MVB (multivesicular body) formation, that the final envelopment of HHV-6A occurs at trans-Golgi network (TGN-) or post-TGN-derived membranes, and that enveloped virions are released by the exosomal pathway [42]. We found AL structures in HHV-6A-infected cells and some virions inside AL structures. However, the membranes that surrounded or enwrapped the HHV-6A virions were not derived from AL, because the enveloped capsids in the AL were found inside vacuoles that were further enwrapped by AL membrane. Furthermore, immunostaining with anti-gB and anti-gM antibodies showed that gB and gM were abundant on the nucleocapsid-enwrapping membranes, while they were scarce on the AL, suggesting that the origins of the two membranes were different. Regarding tegumentation, we found tegument-like electron-dense material on the cytosolic side of TGN-derived vacuoles and at other regions on these vacuoles.

A model for the replication cycle of HHV-6A based on published reports is as follows [43] (Figure 1). The HHV-6A ligand, the gH/gL/gQ1/gQ2 complex, binds to its receptor CD46 (1), the other viral glycoprotein(s) (e.g., gB) also binds to unidentified cellular receptor(s) and enters the cell via endocytosis (2). After deenvelopment by fusion between the viral and cellular membranes in the endosomal compartment (3), the incoming nucleocapsid is transported through the cytoplasm to the nuclear pore complex (4), where the viral DNA genome is unpackaged and released into the nucleus (5). In the nucleus, viral gene transcription and genome replication occur (6). Long concatemeric strands of progeny DNA are cleaved to unit lengths and encapsidated (7). The capsids bud into the perinuclear cisternae (8) and acquire a primary envelope in the perinuclear space (9). Deenvelopment of the nucleocapsid occurs in the cytoplasm (10), and reenvelopment occurs by budding into TGN- or post-TGN-derived membranes (11). Finally, the virion-containing vacuoles expand and MVBs are formed (12), and the enveloped virions are released by the exosomal pathway by fusion of the MVBs with the plasma membrane (13).

3.2. HHV-6B. Compared with HHV-6A, fewer reports have been published on the details of HHV-6B replication. In 1990, Nii et al. proposed the envelopment-deenvelopment-reenvelopment pathway model for HHV-6B (Hashimoto), based on examinations of infected MT4 cells and human lymphocytes [44]. Ahlqvist et al. supported this model with observations in Z29-infected SupT-1 cells [37].

Conflicting results had been reported about HHV-6B's tegumentation, as with HHV-6A. Nii et al. found that tegument-coated capsids could be detected only in the cytoplasm, and not in the nucleus or perinuclear cisternae, suggesting that tegumentation occurred in the cytoplasm [44]. However, as described above, Roffman et al. reported that HHV-6B (Z29 stain) acquires its tegument at the tegument compartment [38], and Ahlqvist et al. supported this model by showing that Z29-infected SupT-1 cells form tegusomes [37]. Regarding AL, Ahlqvist et al. reported that no AL could be found in the Z29-infected SupT-1 cells [37].

4. Lipid in the HHV-6A Envelope and Target-Cell Membrane

Our group showed that cholesterol in both the HHV-6A envelope and target cell is required for HHV-6A entry [45,

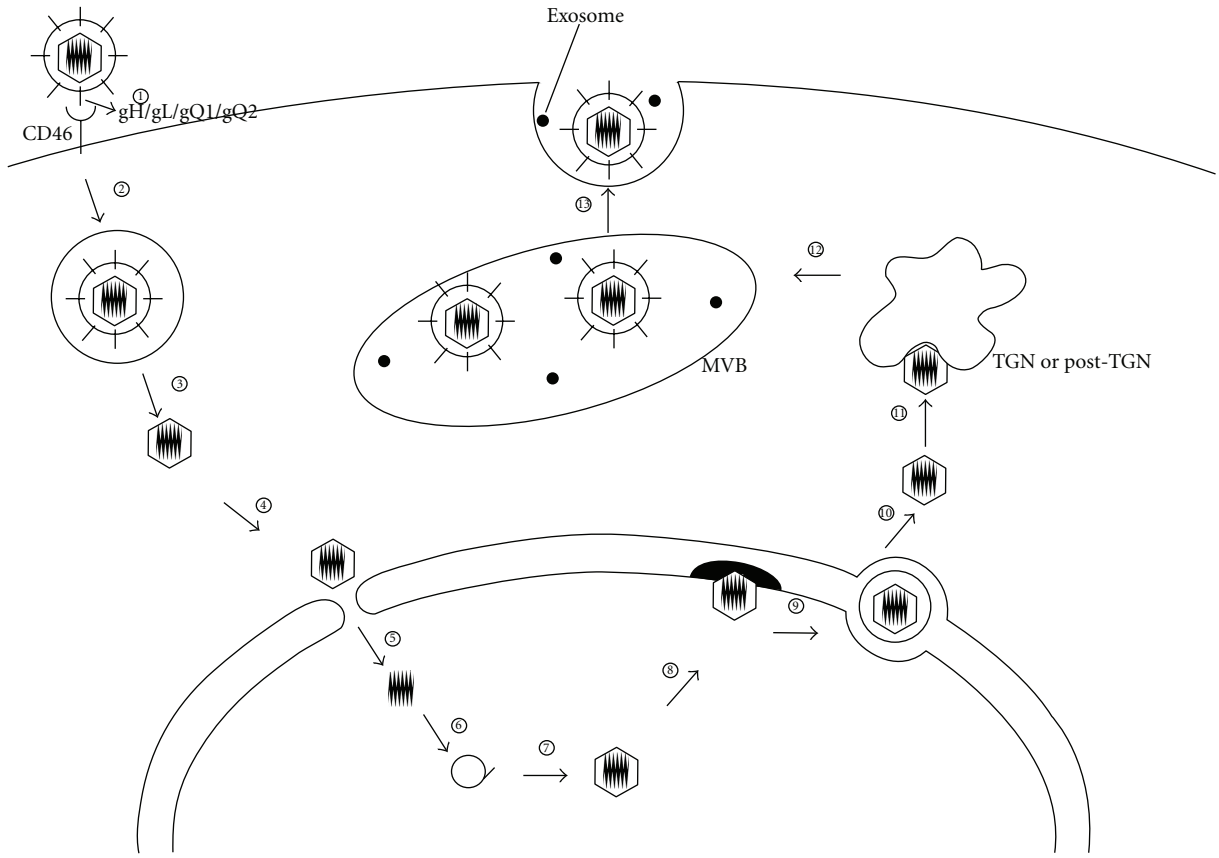

Figure 1: Schematic representation of the HHV-6A replication cycle. A diagram of the proposed replication cycle for HHV-6A is shown. HHV-6A gH/gL/gQ1/gQ2 binds to CD46 (1) and enters the cell via endocytosis (2). The deenveloped nucleocapsid is then transported to the nucleus (3, 4), where the viral genome is released (5). After viral gene transcription and genome replication (6), the progeny DNA is encapsidated (7), and the capsid buds into the perinuclear cisternae (8). The capsid acquires a primary envelope in the perinuclear space (9), and deenvelopment occurs in the cytoplasm (10). The nucleocapsid acquires the final envelope by budding into TGN- or post-TGN-derived membrane (11). Vacuoles containing virions expand and form MVBs (12), then the mature enveloped virions are released via the exosomal pathway (13).

46]. In addition, we showed that HHV-6A infection induces the relocation of CD46 into lipid rafts and that glycoproteins (gQ1 and gB) are associated with lipid rafts, indicating that lipid rafts of the cell membrane are important for viral entry and that HHV-6A may enter the target cells via lipid rafts [46]. Furthermore, we reported that the HHV-6A envelope contains lipid rafts, suggesting that HHV-6A virions might assemble through lipid rafts [47]. These results show that lipid rafts in both the HHV-6A envelope and its target cells play a critical role in HHV-6A entry and might be involved in HHV-6A virus assembly.

5. Glycoproteins That May Be Involved in Cell Fusion

Glycoprotein B (gB), glycoprotein H (gH), and glycoprotein L (gL), are conserved in all herpesviruses, and essential for entry [48]. As in other herpesviruses, gB and gH in HHV-6A and HHV-6B are known targets for neutralizing antibodies, so they are known to be major determinants for cell entry in HHV-6A and HHV-6B [49, 50]. Futhermore, we showed that gB and gH are required for U1102-induced polykaryocyte formation [26].

For other herpesviruses, especially HSV, reports have accumulated about the roles of viral glycoproteins in entry [1, 29, 30, 51–53], and structural analyses of herpesvirus glycoproteins have been performed [54–56]. EBV utilizes gp42 with gH/gL as a switch of cell tropism and the structural analysis of gp42 has also been done [1, 57].

However, in the case of HHV-6A and HHV-6B, the structures of the gH/gL or gH/gL/gQ1/gQ2 complexes have not yet been reported, and the detailed mechanisms remain unknown. To unveil the entry mechanisms in detail, determination of the gH/gL and gH/gL/gQ1/gQ2 structures and detailed analyses of each glycoprotein's function are needed.

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