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*Review Article*

# Molecular Signaling and Cellular Pathways for Virus Entry

**Pei-I Chi**[1] **and Hung-Jen Liu**[1,2]

[1] *Institute of Molecular Biology, National Chung Hsing University, Taichung 402, Taiwan*
[2] *Agricultural Biotechnology Center, National Chung Hsing University, Taichung 402, Taiwan*

Correspondence should be addressed to Hung-Jen Liu; hjliu5257@nchu.edu.tw

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The cell signaling plays a pivotal role in regulating cellular processes and is often manipulated by viruses as they rely on the functions offered by cells for their propagation. The first stage of their host life is to pass the genetic materials into the cell. Although some viruses can directly penetrate into cytosol, in fact, most virus entry into their host cells is through endocytosis. This machinery initiates with cell type specific cellular signaling pathways, and the signaling compounds can be proteins, lipids, and carbohydrates. The activation can be triggered in a very short time after virus binds on target cells, such as receptors. The signaling pathways involved in regulation of viral entry are wide diversity that often cross-talk between different endocytosis results. Furthermore, some viruses have the ability to use the multiple internalization pathways which leads to the regulation being even more complex. In this paper, we discuss some recent advances in our understanding of cellular pathways for virus entry, molecular signaling during virus entry, formation of endocytic vesicles, and the traffic.

## 1. Introduction

Unlike the other organisms, in order to create its progeny, viruses need the hosts to provide the replicate resources. In order to deliver their genomes into the host cells for their own purposes, viruses have to overcome the barrier of the cell, the plasma membrane. There are several viral entry ways that have been identified, like genetic injection including phages and membrane fusion such as human immunodeficiency virus type 1 (HIV-1) [1]. The cell endocytic mechanism also provides a route for virus internalization. Recent research has discovered that numerous viruses favor the endocytosis as clathrin-mediated endocytosis (CME) [2–7], caveolae/lipid raft-mediated endocytosis [8–10], macropinocytosis [11–13], and several other unusual pathways [14, 15] by eliciting the endocytic signaling pathways. This review focuses on the elements that are involved in regulating the mechanism of virus entry and their traffic systems.

## 2. Clathrin-Dependent Endocytosis

Although many viruses had shown that their internalization relied on the CME [2–7], the signals that are triggered by viruses are more complicated for their own benefits. Many viruses are low-pH-dependent for their conformation change [16, 17] that is required for membrane fusion or viral particle uncoating, and the endosomes provide this acidic compartment. Therefore, the viruses that entry the host cells via clathrin-mediated endocytosis in most cases will form the clathrin-coated vesicles (CCVs) by triggering the clathrin to the plasma membrane. Ap-2, the plasma membrane adaptor complex which recruits clathrin and are Eps15 that required in the CME [18, 19]. Ap-2 also bind to amphiphysin I, amphiphysin II, epsin and further interact with other endocytic proteins, like GTPase dynamin, synaptojanin and intersectin [20]. Dynamins are required in the process of clathrin-coated vesicles (CCVs) fission form the plasma membrane [21]. The entry procedure of most enveloped viruses involves endocytosis and membrane fusion. Dynamin has been suggested to act both as a regulatory GTPase by controlling the early stages of CME, which is an important endocytic pathway used by many viruses, and as a chemical enzyme that induces membrane fission and pinches endocytic vesicles off from the cellular plasma membrane in later stages in several endocytic pathways, including CME. In addition to its involvement in virus entry,

dynamin has also been proposed to participate in membrane fusion between the virus and endosomes following endocytosis [22]. Besides, the actin dynamics have been discovered in regulating multiple endosome steps in the Kaposi's Sarcoma-associated herpersvirus (KSHV) entry and traffic systems. By inhibiting Src, Rho GTPases, and Arp2/3 which are required for actin nucleation, results in blocking the KSHV entry and trafficking [23, 24].

Clathrin-dependent entry mostly follows the route from early endosomes (EE) to late endosomes (LE), and further to lysosomes for degradation or conformation changes. To deliver the cargo from CME to EE only require 2–5 min, and take another 10–15 min to reach the LE [25]. These CCVs to LE through EE is fast and mostly in Rab GTPase family-dependent and associated with PI3-kinase [26]. The CME of borna disease virus (BDV) requires Rab5 and microtubules [27]. However, the actin dynamics were not necessary in the BDV vial entry process. Rab5 and EEA-1 are associated with the process in transporting the cargo from CCVs to EE and maturing endosome (ME), and dissociated when transport to LE. Although it is not always require Rab7 in viral entry mechanisms, but in some viruses like Influenza A virus and semliki forest virus (SFV), are associated with the sorting and transport to LE [28, 29]. Vps27/Hrs recruits endosomal sorting complex required for transport (ESCRT) complex to sort cargos that tagged with ubiquitin, results with the LE lumen filled with intraluminal vesicles (ILV) by invagination process, and destine for degradation while transport to lysosomes [30, 31]. The lumens become more acidic from LE to lysosomes through V-ATPase regulation [32] and provide the environment for viruses partial disassemble or envelope fusion with the vesicle membrane that benefits for release the viral proteins before degradation by lysosomes. However, the viruses like parvoviruses required further traffic to lysosomes [33]. Some viruses might involve in more than two endocytosis pathways [34–36]. The varicella-zoster virion (VSV) does not only trigger clathrin-mediated endocytosis but also required the cholesterol, the component of lipid raft [37].

## 3. Caveolae/Lipid Raft-Dependent Endocytosis

Caveolae is morphologically distinct entities that organize lipid and protein components. Caveolae, a subset of membrane (lipid) rafts, are flask-like invaginations of the plasma membrane that contain caveolins, which serve as organizing centers for cellular signal transduction. Caveolae consist of caveolins, 20 kDa caveolae-resident proteins with a unique hairpin structure and cytoplasmic amino and carboxyl termini. Although caveolins were named based on their identification in caveolae, they differ in their patterns of expression in different cell types [38] and are also expressed in other cellular locations [39]. Caveolins possess the caveolin scaffolding domain (CSD) to which signaling molecules bind in an inactive state; activation leads to conformational changes, thereby releasing and activating the signaling proteins [40, 41]. Caveolin and certain binding partners interact with the CSD. The CSD is a peptide sequence that contains binding motifs that scaffold signaling molecules: adenylyl cyclase (AC), protein kinase A (PKA), protein kinase C (PKC), heterotrimeric G$\alpha$ and G$\beta\gamma$, Src, phosphatidylinositol 3-kinase (PI3 K), endothelial nitric oxide synthase (eNOS), and mitogen activated protein kinase.

In addition to CME, the mammalian cells possess the clathrin-independent endocytosis; one of them is the lipid raft-dependent endocytosis. Lipid rafts are enriching of membrane microdomains with glycosphingolipids and cholesterol, and acts as platforms for protein trafficking and signal transduction. Although cholesterol is the component of lipid rafts, but exceeding amount of cholesterol will hinder the entry of the viruses, such as Japanese encephalitis virus (JEV) and dengue virus serotype 2 (DEN2) [42]. The primary endocytic vesicles formation in caveolae/lipid raft-dependent pathway normally depends on the phosphatases, Src family kinases, tyrosine kinases, G protein-coupled receptors (GPCRs), integrins and ligand-triggered signaling pathways that associated with cholesterol and lipid rafts [43]. This route of endocytosis share some mechanisms similar to CME but differ in their molecular factors.

Viral infections are known to activate various cellular signaling pathways to facilitate replication. An increasing amount of information has demonstrated that numerous viruses activate the p38 MAPK pathway to enhance their efficient replication [44–48]. Uptake may involve the lipid raft-containing microdomains as caveolae and dynamin 2. Simian virus 40 (SV40), one of a typical example which induces internalization is ligand-triggered caveolae dependent. SV40 had shown to recruit dynamin and rearranges the actin cytoskeleton during its endocytosis [49]. The echovirus 1 (EV1) endocytic internalization together with its receptor $\alpha$2 and $\beta$1 integrin, the caveolin-1 associated elements, that forms caveosomes in lipid raft, dynamin 2-PKC$\alpha$ and ERK-dependent manner [50, 51]. Similar signaling regulation is also seen in avian reovirus (ARV). ARV has been reported to activate Ras, p38 MAPK, and src followed with caveolin-1 mediated endocytic pathway in dynamin-2 dependent [10]. ARV-induced phosphorylation of caveolin-1 (Tyr14) and dynamin-2 expression as well as Rac1 activation through activation of p38 MAPK and Src in the early stage of the virus life cycle is beneficial for virus entry [10]. It was demonstrated that both p38 MAPK and Src are associated with cellular proteins caveolin-1 and dynamin-2. A novel role for p38 MAPK and Src signaling has been found to exert positive effects on caveolin 1 and dynamin 2 expressions, thereby enhancing ARV entry [10]. ARV traffics to early endosomes require the participation of microtubules and small GTPase Rab5 [10]. In addition to regulation of virus entry, it has also been found that p38 MAPK activation is beneficial for ARV replication [52].

Although the integrins are required in most cases of endocytosis that associated with lipid raft, however, the latest report indicated that west vile virus entry requires cholesterol-rich membrane microdomains and is independent of a$v\beta$3 integrin. Even though, we cannot rule out other integrins might involve in the internalization of MNV [53]. The route of caveosomes passes through endosome traffic pathway from EEs to LEs, and generally, further to endoplasmic reticulum (ER) in vesicle-mediated transport system

which followed with the viral uncoating and penetration into the cytosol [54, 55]. This route requires longer time for virus's traffic to their destinations compared with CME, as dynamin and caveolae associated with the additional level of regulation. After reaching ER, viruses accumulate in smooth membrane network and tubular, followed with microtubule-mediated movement in both plus and minus end directions, then further deliver to cytoplasm or nucleus dependent on the virus types [25, 56]. Some viruses are not lipid rafts essential for their binding and entry, but will require those lipid rafts-induced pathways for their further works. For example, KSHV enters host cells mainly through CME but triggers FAK, Src, PI3 K, RhoA, Dia-2, Ezrin, PKCζ, and Diaphanous-2 (Dia-2) during its infection [36, 57].

## 4. Macropinocytosis

Cell entry of viruses that rely on macropinocytosis will be "engulfed" by the cell membrane formed with outward extension membrane ruffles fold back, similar to phagocytosis. Therefore, macropinocytosis mechanism requires actin cytoskeletal reorganization. Virus entry with this route needs to trigger the actin modulatory factors, and often with the activation of PI3 K and Rho GTPases Rac1, Cdc42, and other cellular kinases [58]. Binding of viral envelope glycoprotein (Env) of HIV-1 with the primary receptor CD4 and one of two coreceptors, CXCR4 or CCR5, has been found to activate a signaling cascade resulting in Galphaq-mediated Rac activation and actin cytoskeleton rearrangements necessary for HIV-1-mediated membrane fusion [59]. HIV-1 employs envelope-CXCR4 interaction to activate a cellular actin depolymerization factor, cofilin, to support viral latent infection of resting CD4 T cells [60]. It has also been uncovered that Env-mediated cell-cell fusion, virus-cell fusion, and HIV-1 infection are dependent on Tiam-1, Abl, IRSp53, Wave2, and Arp3 [61].

Macropinocytosis can be induced by growth factors and forms macropinosomes. More recently, Diao and colleagues demonstrated that direct hepatitis C virus (HCV) binding to CD81 can induce EGFR activation and internalization [62]. HCV interaction with CD81 can also activate multiple downstream signaling pathways including Rho GTPase family members, Cdc42, MAPK pathways, and members of the ezrin-radixin-moesin (ERM) family of proteins [63–65]. EGFR has also been demonstrated to play a critical role in the entry process of other viruses, including influenza A virus, human cytomegalovirus (HCMV), and adeno-associated virus serotype 6 (AAV6) [66, 67]. Interestingly, EGFR activation was demonstrated to be required for influenza A virus internalization via the clustering of lipid rafts [67], implicating that EGFR internalization may be a common mechanism used by viruses to enter their host cells.

Macropinosome vary in size and its acidic environment is beneficial for viruses that are low-pH-dependent in their life cycles [68]. Under different signaling regulations, the macropinosomes can fused with EE or recycled back to plasma membrane. To date, several viruses have been found to enter their host cells by macropinocytosis, including vaccinia virus, HIV-1, coxsackievirus B (CVB), herpes simplex virus type 1 (HSV-1), african swine fever virus (ASFV), human papillomavirus type 16 (HPV-16), and KSHV [11–13, 69]. Recently, it was reported that vaccinia virus entry relies on type II membrane glycoprotein CD98 associated integrin $\beta$1 triggered PI3 K/Akt signaling and ERK, PKC, and PAK1 that are required for macropinosome closure [70, 71]. HIV-1 requires $Na^+/H^+$ exchange as other macropinocytic viruses, nevertheless, just like vaccinia virus; its endocytosis also depends on activation of the lipid-rafts and MAPK signaling [72, 73]. More recently it was also found that internalization of ASFV depends on actin reorganization, activity of $Na^+/H^+$exchangers, and signaling events typical of the macropinocytic mechanism of endocytosis [12]. Cell entry of virus appears to directly stimulate dextran uptake, actin polarization and EGFR, PI3 K/Akt, Pak1 and Rac1 activation [12]. Interestingly, Schelhaas and colleagues uncovered that HPV-16 utilizes a potentially novel ligand-induced endocytic pathway related to macropinocytosis. This pathway is different from classical macropinocytosis in regards to vesicle size, cholesterol-sensitivity, and GTPase requirements, but similar in the need for tyrosine kinase signaling, PAK-1, PKC, actin dynamics, and $Na^+/H^+$ exchangers [13, 69].

In the case of CVB, its entry and the macropinosomes trafficking are caveolin-depnendent but dynamin independent that require occludin, Ras-Rab5 pathway, and Rab34 activation [74]. In some viruses, internalization does not enter macropinosomes, but the macropinocytosis needs to be triggered for initiating their infectious processes. Adenovirus type 2 (Ad2), as an example, can activate the $\alpha_v$ integrin coreceptors to trigger macropinocytosis that benefit for viral escape from endosomes to cytosol [75].

## 5. Unusual Endocytic Pathways

Apart from the established endocytic pathways described above, there also exists other mechanisms that used by some viruses, like lymphocytic choriomeningitis virus (LCMV), endocytosis is in clathrin, dynamin-2, caveolin, lipid rafts, actin dynamics, Arf6 and flotillin-1 independent, but in membrane cholesterol dependent. The entry of LCMV occurs in noncoated pits and transfers to LE directly without passing through Rab5/EEA1-positive EE [15, 76]. One other example is herpes simplex virus type 1 (HSV-1). Although previous studies had indicated that the endocytosis of HSV-1 involves macropinocytosis [77], but Clement and Rahn have discovered that, the HSV-1, can take another route; a phagocytosis-like uptake that is facilitated by nectin-1, dynamin, and cholesterol-dependent manner [78, 79]. Influenza virus as mentioned above, enter host cells mainly via clathrin-mediated endocytosis. However, the other endocytic route has also been discovered to provide an additional entry pathway independent of clathrin and caveolae [34]. It is believed that there exist other novel pathways not been described yet, and they might serve as important entry routes for those clathrin-independent and caveolae-independent viruses.

## 6. Integrin, Chemokine, and Heparan Sulfate Receptors-Mediated Endocytosis

The cell surface organization of receptors and their signaling partners have been the topics of long-standing interest in several biological disciplines, including biochemistry, cell biology, physiology, and pharmacology. Recent data have emphasized the importance of colocalization of receptors with their signaling partners in discrete microdomains so as to facilitate the activation of cellular events. Integrins are the major cell surface adhesion receptors for ligands in the extracellular matrix (ECM). They are heterodimeric proteins consisting of an alpha- and a beta-chain involved in the transmission and interpretation of signals from the extracellular environment into various signaling cascades [80]. Several recent reports have provided mechanistic insight into how integrin traffic is regulated in cells. Increasing evidences indicates that small GTPases such as Arf6 and members of the Rab family control integrin internalization and recycling back to the plasma membrane along microtubules [81]. Several studies have suggested that integrins are involved in virus entry [82–86]. There are several examples of signaling mediated by the integrin. More recently, Lzmailyan and colleagues have uncovered that integrin $\beta 1$ mediates vaccinia virus entry through activation of PI3 K/Akt signaling [70]. In the case of KSHV, it utilizes heparan sulfate-like molecules to bind the target cells via its envelope-associated glycoproteins gB and gpK8.1A [87]. The envelope glycoprotein gB of KSHV possesses an Arg-Gly-Asp (RGD) motif that interacts with $\alpha 3\beta 1$ integrin as one of the receptors for its entry into the target cells via its gB delta transmembrane (TM) interaction and induces the activation of focal adhesion kinase (FAK) [82]. FAK activation is a critical step in the outside-in signaling pathways necessary for the subsequent phosphorylation of other cellular kinases, cytoskeletal rearrangements, and other functions. FAK was critical for the subsequent phosphorylation of Src by gB delta TM, and Src induction is crucial for the phosphorylation of PI-3 K. KSHV gB delta TM-induced PI-3 K is essential for the induction of RhoA and Cdc42 Rho GTPases that is accompanied by the cytoskeletal rearrangements. In case of DENV-2, $\alpha 5\beta 3$ integrin has been identified in endothelial cells (HMEC-1) as a putative receptor for the virus. The actin cytoskeleton of HMEC-1 cells plays an important role in virus entry and infection. Internalization of DENV-2 into endothelial cells requires viral induction of dynamic filopodia regulated by Rac1 and Cdc42 cross-talk and myosin II motor activities [88].

Adenoviruses are a significant cause of acute infections in humans. A greater understanding of the highly effective adenovirus cell entry pathway may lend itself to the development of safer drug and gene delivery alternatives utilizing similar pathways. The penton and fiber capsid proteins of adenovirus mediate infection and interact with cellular proteins to coordinate stepwise events of cell entry that produce successful gene transfer [83]. The fiber initiates cell binding while the penton binds alpha (v) integrin's coreceptors, triggering integrin-mediated endocytosis. Penton integrin signaling precedes viral escape from the endosomal vesicle. Intracellular trafficking of the remaining capsid shell is mediated by the interaction of naked particles with the cytoskeleton. Adenovirus receptor (CAR) is an attachment receptor for adenovirus serotype 5 (Ad5) and in many cell types forms homodimers with neighboring cells as part of a cell adhesion complex. CAR co-operates with cell surface integrin receptors to enable efficient viral entry [84]. CAR-induced p44/42 MAPK activation leads to increased activation of $\beta 1\beta 3$ integrins, suggesting that signaling downstream of CAR has a dual effect on integrins and CAR itself in order to promote efficient viral binding to cell membranes.

Viruses have evolved a number of strategies to gain entry and replicate in their host cells that, for HIV and the poxvirus, myxoma virus, involve appropriating chemokine receptors [89]. The current general model of HIV entry involves the binding of viral envelope glycoprotein gp120/gp41 to cell surface receptor CD4 and G-protein-coupled chemokine coreceptor CXCR4 or CCR5, which triggers conformational changes in the envelope proteins [89]. In addition to the CCR5 and CXCR4 chemokine receptors, a subset of primary HIV-1 isolates can also utilize the seven-transmembrane-domain receptor APJ as a coreceptor [90]. A complete model of HIV-1 entry includes the involvement of membrane microdomains, actin polymerization, glycosphingolipids, and possibly CD4 and chemokine signaling in entry.

Heparan sulfate, a member of the glycosaminoglycan family, normally exist as proteoglycan (HSPG) form and attaches to the extracellular matrix protein on the cell surface. As described earlier, the KSHV uses heparan sulfate-like molecules to bind the target cells [87], many microorganisms or viruses like Rift Valley fever virus (RVFV), herpesviruses, and adeno-associated virus (AAV) also utilize this structure as a docking site to the target cells [91–93]. After binding to the heparan sulfate, the virus can interact with other receptors on the surface to initiate its endocytosis or membrane fusion [92]. Although a previous report demonstrated that herpesvirus infection can rapidly upregulate the express level of heparan sulfate on the B-cell membrane by activating type I IFN [94], however, the signaling that benefits virus entry after binding on heparan sulfate still remains unclear.

## 7. Conclusions

To infect the cell, viruses must bind to cell surface, followed with signaling induction, in order to penetrate into their host cells. Cell membrane is a barrier for outcoming sources, which include viruses. Virus penetration can take either by fusing with the plasma membrane directly to release viral capsids into cytosol or by endocytosis [95]. Virus entry often uses multiple pathways and is cell type specific, due to the components diversity in either membrane or organelles that might limit the virus entry ability. Besides, viruses that differ in types might also trigger distinct routes for entry, as human papillomavirus (HPV) type 31 and type 16 [96]. The signaling triggered by viruses or the endocytic effect is even more complex. Clarification of pathways and mechanisms for virus entry will benefit drug development for specific anti-virus treatment.

## Conflict of Interests

The authors declare no conflict of interests.

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