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Endocytosis of influenza viruses

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Abstract

Receptor-mediated endocytosis is known to play an important role in the entry of many viruses into host cells. However, the exact internalization mechanism has, until recently, remained poorly understood for many medically important viruses, including influenza. Developments in real-time imaging of single viruses as well as the use of dominant negative mutants to selectively block specific endocytic pathways, have improved our understanding of the influenza infection process.

Keywords

Influenza; endocytosis; clathrin

1. Introduction

Viruses must deliver their genome into the host cells to initiate replication. While some virus species can directly penetrate the plasma membrane and inject their genetic material into the cytoplasm (e.g. HIV), the majority of viruses enter cells via endocytosis. The latter strategy enables a virus to hijack the cell's own machinery to be transported past the plasma membrane and through the cytoskeleton. Endocytosed viruses are first internalized into intracellular compartments, such as endosomes, and then penetrate the endosomal membrane to release their genome. This membrane penetration is often triggered by the acidic milieu of the endosomes. For enveloped viruses, this process occurs via a protein-catalyzed membrane fusion process between viruses and endosomes. Non-enveloped viruses, on the other hand, lyse the limiting membrane of endosomes or generate a pore on the membrane so that the viral genome can enter the cytoplasm. For some viruses, such as influenza, an additional barrier exists for successful gene delivery - the genome has to be imported into the nucleus for replication and expression.

Due to the presence of multiple endocytic pathways in cells, determining the exact endocytic mechanisms used by viruses has been challenging[1,2]. Cellular endocytosis includes phagocytosis and pinocytosis[3,4]. Phagocytosis is typically restricted to specialized cells such as macrophages and is mainly employed to digest large particles and bacteria. Pinocytosis is responsible for the cell's uptake of fluids, macromolecules and small pathogens such as viruses. Pinocytosis can occur by several different mechanisms (Fig. 1): macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis, and clathrin- and caveolae-independent pathways[3,4]. Macropinocytosis is often associated with growth factor-induced membrane ruffling and protrusion, which then collapse to form endocytic vesicles, referred to as macropinosomes[3,4]. Clathrin-mediated endocytosis occurs by the concentration of receptors and bound ligands into clathrin-coated pits (CCPs), which pinch off from the plasma membrane

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to form clathrin-coated vesicles (CCVs)[3-9]. These CCVs then uncoat and fuse with other endocytic vesicles or endosomes. Caveolin-mediated endocytosis occurs via caveolae, i.e. caveolin-associated membrane invaginations, that pinch off to form endocytic vesicles and fuse with caveolin-containing membrane compartments, often referred to as caveosomes[3,4,10,11]. Other clathrin- and caveolin-independent pathways are known to exist but are poorly understood due to the lack of known marker proteins[3,4,12-16].

Although viruses may be taken up by one or more of the above mechanisms, not every pathway necessarily leads to successful infection. To get an accurate view of the situation, it is necessary to employ experimental techniques that can isolate individual virus-entry events or specific endocytic pathways and discriminate ones that lead to infection. Recent developments in molecular biology and in optical microscopy techniques have made this possible. For example, the construction of dominant-negative mutants that selectively inhibit specific endocytosis mechanisms has provided new insights into the endocytic mechanisms of several viruses[1,2,17-19]. The development of high-sensitivity video microscopy and natural fluorescent proteins has allowed the imaging and tracking of single endocytic structures[10,20-22] and single virus particles[10,23-29] in living cells, providing a direct means of observing which endocytic pathways are involved. Because single-virus tracking experiments are able to follow individual virus particles in real-time, they can also provide information about how virus particles are targeted to endocytic structures as well as the dynamics of post-endocytic trafficking[10,24-29]. These techniques have the power to detect previously unknown endocytosis mechanisms, as was recently demonstrated for the caveolae-dependent entry of SV40 virus [10]. In this review, we focus on influenza virus[19,28-32].

2. Influenza virus

Influenza has long been used as a model system to understand viral entry. It is an enveloped virus with a lipid-bilayer membrane and three types of integral membrane proteins, Hemagglutinin (HA), Neuraminidase (NA) and M2 (Note: type C influenza has only a single glycoprotein, the hemagglutinin-esterase-fusion protein while influenza A and B have both HA and NA) [33]. Beneath the lipid membrane is a protein matrix made of M1[33]. A segmented genome, comprised of eight single-stranded RNAs packed into ribonucleoprotein (vRNP) complexes, resides inside the virus[33]. The protein components of the vRNPs are the nucleoproteins (NP) and RNA-polymerases (PA, PB1 and PB2)[33]. Other proteins encoded by the influenza genes include NS1 and NS2[33].

Influenza viruses infect cells in a multi-step process (Fig. 2): (i) viruses are internalized via receptor-mediated endocytosis; (ii) internalized viruses are trafficked along the endocytic pathway to acidic late endosomes; (iii) exposure to low pH triggers HA-catalyzed fusion between the viral and endosomal membranes, releasing vRNPs; (iv) vRNPs are imported into the nucleus for viral gene expression and replication[1,2,33-35]. Here we focus on the first two steps - endocytosis and post-endocytic trafficking of influenza.

3. Endocytosis mechanisms of influenza viruses

Initial insights into the influenza entry pathway came from electron microscopy[30,36]. Electron micrographs of influenza virus-infected cells have shown viruses inside coated pits on the cell surface and coated vesicles inside the cell[30]. These results provide direct evidence that influenza viruses can enter cells via clathrin-mediated endocytosis. However, in these experiments virus particles were also found inside smooth, uncoated surface invaginations and vesicles[30]. In contrast, Semliki Forest virus (SFV)[37] and vesicular stomatitis virus (VSV) [30] were not found in smooth pits and vesicles in similar electron microscopy experiments. These observations seem to indicate that while SFV and VSV enter cells via clathrin-mediated endocytosis only, influenza may exploit an additional, clathrin-independent endocytic

mechanism. However, without the ability to follow the time course of individual viral particles, it is not known whether these smooth pits lead to successful influenza entry, nor is it clear whether the smooth virus-bearing vesicles result from clathrin-independent endocytosis or from uncoating of CCVs after clathrin-dependent entry.

A recent study tested the infectivity of influenza viruses in the presence of a dominant negative mutant of Eps15, Eps15 Δ 95/295, which specifically blocks clathrin-mediated endocytosis [19]. Eps15 is an essential component of CCPs, associated with the AP2 complex. Infectivity was tested by probing the synthesis of influenza NP in virus-inoculated cells. Surprisingly, influenza infectivity was virtually unimpaired in cells expressing Eps15 Δ 95/295 (Fig. 3a,b) whereas control experiments with the same mutant effectively inhibited infection with SFV [19]. Influenza also retained its infectivity in the presence of chlorpromazine, a drug that blocks clathrin-mediated endocytosis. These results indicate that influenza viruses can indeed infect cells via a clathrin-independent pathway under certain conditions.

In addition, influenza infectivity was essentially unaffected by the cholesterol-sequestering drugs nystatin and methyl- β -cyclodextrin, that are known to inhibit caveolin-mediated endocytosis (Fig. 3c)[19]. Infection was also not substantially impaired in cells expressing a dominant negative mutant of caveolin-1. In fact, influenza can enter and replicate in cells even in the presence of both Eps15 Δ 95/295 and cholesterol-sequestering drugs, with only a moderate decrease in infectivity[19]. These results indicate that influenza can infect cells via a clathrin- and caveolin-independent endocytic pathway(s). The possibility remains, however, that expression of dominant negative mutants leads to an upregulation of other endocytic processes that are not used by influenza under unperturbed cellular conditions. As a result, the relative significance of the clathrin and caveolin independent pathway(s) in influenza infection is unclear.

We have recently studied the entry mechanism of influenza by directly monitoring the interaction between single viruses and cellular endocytic machinery such as CCPs and caveolae in real-time in living cells[29]. This has allowed us to characterize the endocytic mechanism exploited by each virus without having to inhibit specific endocytic pathways. We fluorescently labelled individual CCPs by expressing in live cells a chimeric fusion protein consisting of clathrin light chain *a* and enhanced yellow fluorescent protein (EYFP) [20,21]. Similarly, we labelled individual caveolae using a fusion protein consisting of caveolin-1 and green fluorescent protein (GFP)[10,22].

Real-time imaging and tracking of individual dye-labeled viruses in living cells revealed that the internalized viruses undergo microtubule-dependent movement prior to achieving viral fusion[28,29]. Using the onset of microtubule-dependent movement as a indication that viral internalization has occurred, we have found that about two thirds of the endocytosed virus particles associate with CCPs for an extended period of time prior to internalization (Fig. 4a,b), indicating the endocytosis of these viruses via the clathrin-mediated pathway[29]. The remaining one third did not show any association with a clathrin-containing structure prior to internalization (Fig. 4c), while a control experiment with transferrin - a classical marker for clathrin-mediated endocytosis - indicated that over 96% of the CCPs were visible in our experiments. These results suggest that one third of the viruses were internalized via a clathrin-independent pathway[29]. By tracking single virus particles together with caveolae, we have found the clathrin-independent pathway(s) to be also caveolin-independent [29].

Upon exposure to low pH ($\sim$ 5), influenza viruses fuse with their endosomal compartments, allowing their genome to be released into the cytoplasm to initiate infection[2,30,34,38]. Using a fluorescence dequenching-based probe, we were able to detect individual virus fusion events [28]. Considering only the subset of viruses that successfully fused, we have shown that the

partition between the clathrin-dependent and clathrin- and caveolin-independent entry was nearly identical to that for all viruses that show microtubule-dependent motion[29]. These results indicate that the clathrin-mediated and -independent pathways are equally efficient for viral fusion once the viruses are internalized. Most likely, these pathways are also equally efficient for infection, considering that influenza is highly infectious in the presence of drugs and mutants that block clathrin-mediated endocytosis[19]. Since these single-virus tracking results do not rely on any disruption of cellular function, they also show that clathrin- and caveolin-independent endocytosis is not an alternative means exploited by influenza only when clathrin-dependent endocytosis is blocked, but a parallel pathway that influenza takes under normal cellular conditions.

4. Dynamics of influenza endocytosis

Another important question in viral entry is how viruses are targeted to endocytic machinery for internalization. This is in fact a largely open question for many other endocytic ligands as well. For clathrin-mediated endocytosis, two different targeting mechanisms may be possible: 1) ligands and bound receptors may be targeted to pre-existing CCPs on the cell surface, 2) clathrin and cofactors may be recruited to the site of the bound ligand, leading to *de novo* formation of a CCP at that site. The first mechanism has been directly observed for G-protein coupled receptors[39,40].

The ability to monitor individual viruses in real time makes the single-virus tracking approach particularly well suited to study endocytic dynamics and to directly observe how viruses are targeted to endocytic machinery. Using this approach, we found that the influenza virus particles entering via the clathrin-mediated pathway were predominantly (94%) internalized via the *de novo* formation of CCPs at the virus-binding sites[29]. The *de novo* formation of CCPs around viruses has also been recently observed during the cellular entry of reoviruses (personal communication, Tom Kirchhausen and Marcelo Ehrlich).

Real-time observation of the entire formation and disassembly process of CCPs/CCVs around the influenza viruses led to a quantitative characterization of the dynamics of the process[29]: The formation of CCPs begins on average 3 minutes after the viruses bind to the cell. The clathrin signal persists for only about one minute, after which the clathrin coat rapidly disassembles. Shortly after the vesicle has uncoated, the virus begins to undergo microtubule-dependent transport. Interestingly, the formation rate of CCPs at the sites where viruses are bound is much higher (by roughly a factor of 20) than at random sites on the cell surface or sites where a CCP had previously formed[29]. The formation kinetics of CCP at the sites of bound viruses suggest that the *de novo* formation of CCPs there is most likely induced by viral binding[29].

While ligand-induced clathrin-redistribution to the plasma membrane has been previously reported for epidermal growth factor (EGF) and nerve growth factor (NGF)[41], these cases involve global changes in the distribution of clathrin in the cell. The binding of EGF to its receptor causes phosphorylation of the clathrin heavy chain and a global redistribution of clathrin to the cell periphery[42]. The majority of newly induced CCPs do not actually colocalize with the NGF receptors[43,44]. This is distinct from our observation of *de novo* formation of CCPs specifically around bound virus particles without a global redistribution of clathrin[29]. An interesting question arises as to how the signal of viral binding is transmitted across the plasma membrane to initiate the formation of a CCP at the binding site. This is particularly puzzling considering that influenza viruses are generally believed to bind to sialic acids of glycolipids and glycoproteins on the cell surface instead of specific receptors with known internalization motifs. While possible mechanisms for the induced CCP formation may

stem from the highly multivalent binding of influenza to the cell, a detailed understanding is still missing.

IV. Post-endocytic trafficking of influenza

The endocytic system is comprised of a complex, dynamic network of endocytic compartments called endosomes. After internalization, influenza viruses are thought to be trafficked to late endosomes where the acidic environment (pH ~5) triggers fusion and subsequent delivery of the genome to the cytoplasm[1,2,30,38]. Before reaching late endosomes, influenza viruses are believed to undergo at least one prior acidification step upon entry to early endosomes (to pH ~6).

These endocytic compartments, which play an important role in influenza infection, are regulated by Rab proteins and other factors[45-47]. At early endosomes, internalized materials are sorted towards different destinations: recycled ligands and receptors are returned back to the cell surface, while material destined for degradation is sent to lysosomes[45]. Rab5 proteins associate with early endosomes and regulate early endocytic traffic[48-50]. Some ligands and receptors that are destined to be returned to the cell surface are found to reside in distinct recycling endosomes[45-47] that are regulated by Rab11 and possibly by Rab4[50-55]. Likewise, receptors and ligands on the degradation pathway often reside in late endosomes prior to reaching lysosomes. These late endosomes were found to associate with Rab7 and Rab9 proteins[46,56,57].

Several Rab proteins are essential to the trafficking of influenza[31]. Dominant negative mutants of either Rab5 or Rab7 (Rab5 S43N and Rab7 T22N, respectively) significantly inhibit influenza infectivity in HeLa cells, suggesting that both early and late endosomes are required for influenza infection[31]. In contrast, infection with SFV and VSV was inhibited by Rab5 S43N, but was unaffected by Rab7 T22N, consistent with previous results that these viruses can fuse with early endosomes. Using indirect immunofluorescence assays, it was found that influenza viruses colocalize with early endosomal markers at approximately 10 min post infection in HeLa cells[31], while colocalization with late endosomal markers in HeLa cells occurs at approximately 40 min post infection[31]. These results directly indicate the involvement of early and late endosome in influenza entry. However, the entry kinetics obtained in these studies are significantly slower than previous results obtained in MDCK and CHO cells, where the viruses were found to fuse with endosomes at about 10 min post infection [28,38,58]. Single-virus tracking experiments show that trafficking in HeLa cells is indeed substantially slower than in CHO cells[28]. This may be in part responsible for the inefficiency of influenza infection in HeLa cells [59,60].

A recent study found that treatment of cells with the proteasome inhibitor MG132 dramatically reduces influenza infectivity[32]. In the presence of MG132, the viruses seem to be arrested in cellular compartments that do not show substantial colocalization with Rab4, Rab5, Rab7 or Rab9, suggesting that virus particles are trapped in an intermediate compartment that is distinct from the classical early and late endosomes[32]. These compartments also do not colocalize with Rab11, a recycling endosome marker. In contrast, substantial colocalization was found between these virus-containing compartments and an alternative endocytic marker Rme-1 that is thought to associate with some sorting and recycling endosomes[32]. These findings suggest the involvement of the ubiquitin-vacuolar protein sorting system in the influenza infection, although a mechanistic picture of the sorting process is still missing. The requirement of the ubiquitin sorting system appears to be quite specific, as MG132 does not inhibit the infection of SFV and VSV.

Recent single-virus tracking experiments have also provided new insights into the post-endocytic trafficking of influenza viruses. By real-time imaging of dye-labeled viruses in living CHO and BS-C-1 cells, we have observed a three-stage transport behavior prior to fusion that is highly reproducible: the virus first moves slowly in the cell periphery (stage I), then adopts a rapid and unidirectional movement towards the nucleus (stage II), followed by an intermittent, often bi-directional movement in the perinuclear region (stage III) (Fig. 5a,b)[28]. Analysis of virus transport in the presence of actin- and microtubule-disrupting drugs and anti-dynein antibodies indicated that stage I movement is actin-dependent, stage II movement is directed by dynein on microtubules, and stage III involves both minus- and plus-end-directed motilities on microtubules[28]. While stage II and III represent the movement of virus-containing vesicles or endosomes inside the cell, stage I occurs at least in part on the cell surface[28, 29].

Tracking individual viruses co-labeled with pH-sensitive and pH-insensitive dyes revealed a surprising result: the majority of viruses experience their initial acidification step to pH 6 in the perinuclear region after the rapid, microtubule-dependent stage II movement (Fig. 5c,d). The average time elapsed between stage II movement and initial acidification is 0.5 min. Virus fusion appears to occur significantly later than the initial acidification, indicating that an additional acidification step (presumably to late endosomal pH) is required for fusion. These results challenge a previously suggested picture that early endosomes, which are usually distributed in the cell periphery and reached without microtubule-dependent transport, are the early acidification sites of endocytic cargo[61]. Two alternative scenarios seem likely: (i) while the viruses may indeed enter early endosomes before the microtubule-dependent transport, the virus-containing vesicles bud from early endosomes before their acidification to pH 6, and the acidification of the viral cargo itself occurs largely in the perinuclear region following the stage II movement; or (ii) the viruses enter early endosomes in the perinuclear region only after the microtubule-dependent movement. We expect that real-time imaging of individual influenza viruses simultaneously with specifically marked early endosomes can unambiguously distinguish these two scenarios.

V. Conclusion

Recent developments of real-time single-virus imaging techniques and of dominant-negative mutants that selectively inhibit specific endocytic pathways have led to significantly improved understanding of the endocytosis and post-endocytic trafficking of influenza viruses in cells. We now know that influenza viruses infect cells via multiple endocytic pathways, with both clathrin-mediated and clathrin- and caveolin-independent pathway(s) significantly populated. Both pathways lead to viral fusion with similar efficiency once the viruses are internalized. Viruses taking the clathrin-mediated pathway enter cells via the *de novo* formation of CCPs at viral binding sites and the CCP formation at these sites is most likely induced by viral binding. After internalization, the viruses are trafficked via early endosomes to late endosomes, where viral fusion with the endosome leads to the release of viral genome. The transport of viruses to late endosomes occurs in three stages, each with a distinct motor-protein- and cytoskeleton-dependent mechanism. The virus experiences at least two distinct acidification steps, and the first is strongly correlated with the onset of microtubule-dependent transport.

Despite these results, much is still unknown about the influenza infection pathway. Open questions include but are not limited to: How do influenza viruses trigger the formation of CCPs; what are the molecular characteristics of the clathrin- and caveolin-independent endocytic pathway; how are influenza viruses sorted in the endocytic membrane system, and how are they trafficked between different endosomes. Imaging single viruses simultaneously with relevant cellular structures appears to be a promising method to address the above questions, in conjunction with the development of dominant negative mutants or small

interfering RNAs that can specifically inhibit cellular endocytic functions. We also expect this approach to provide new insights into the entry mechanisms of other family of viruses.

Abbreviations Used

CCPs, clathrin-coated pits
CCVs, clathrin-coated vesicles
CHO, Chinese hamster ovary
EYFP, enhanced yellow fluorescent protein
GFP, green fluorescent protein
EGF, epidermal growth factor
HA, hemagglutinin
HIV, human immunodeficiency virus
MDCK, Madin-Darby canine kidney
NA, neuraminidase
NGF, nerve growth factor
NP, nucleoprotein
SFV, Semliki Forest virus
SV40, simian virus 40
vRNP, viral ribonucleoprotein
VSV, vesicular stomatitis virus

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Figure 1.
Possible endocytic pathways exploited by viruses for infection.

Figure 2.

The influenza entry pathway. Influenza viruses bind to receptors containing sialic acid on the cell surface. Virus particles are then endocytosed and enter early endosomes. Subsequently the viruses are trafficked to late endosomes where the low pH triggers viral fusion. Viral ribonucleoproteins (vRNP) escape into the cytosol and are imported into the nucleus, where replication occurs.

Figure 3.

Influenza infection can occur independent of clathrin and caveolin. Cells were infected with ~0.1 PFU of WSN and incubated at 37°C for 4 h. Infection was monitored with immunofluorescence using antibody against the influenza NP. Transfection was monitored with GFP fluorescence. **(a,b)** HeLa cells were transfected with GFP, or GFP-Eps15 Δ 95/295, or left untransfected before infection. **(a)** Immunofluorescence and GFP images of cells infected with WSN. **(b)** Fluorescence activated cell sorter analysis. Infection was monitored on the y-axis and transfection on the x-axis. Labels indicate the percentage of cells within each quadrant. **(c)** Immunofluorescence and GFP images of cells infected with WSN. HeLa cells

were transfected with cav1-GFP(wt) (a fusion protein of GFP with wildtype caveolin-1) or GFP-cav1(DN) (a fusion protein of GFP with the dominant negative caveolin-1). (Figure adopted from Ref.[19])

Figure 4.

Endocytosis of individual viruses. **(a)** Snapshots of a virus internalized via a CCP. Scale bar: 10 μm . $t = 0$ s: the virus (red) binds to the cell. $t = 115$ s: a CCP labeled with EYFP (green) begins to form at the virus site. $t = 175$ s: the clathrin coat rapidly disassembles. $t = 181$ s, 202 s, and 235 s: transport of the virus on microtubules. **(b)** Time-trajectory of a virus internalized via *de novo* formation of a CCP. Black symbols are the instant velocity of the virus. Green symbols are the EYFP fluorescence intensity associated with the virus. Red arrow indicates the onset of microtubule-dependent movement, used as the signal for internalization. **(c)** The time-trajectories of a virus internalized without association with a clathrin-coated structure. Symbols are as defined in **b**. (Figure adopted from Ref. [29])

Figure 5.

Post-endocytic trafficking and fusion of individual viruses. **(a)** The trajectory of a DiD-labeled virus inside a cell. The color of the trajectory codes time with the colored bar indicating a uniform time axis from 0 s (black) to 500 s (yellow). The red star indicates the fusion site. **(b)** Time trajectories of the velocity (black) and the DiD fluorescence intensity (blue) of the virus. The labels I, II, and III indicate stage I (actin-dependent), II (unidirectional, microtubule- and dynein-dependent), and III (bidirectional, microtubule-dependent) movement, respectively. The fluorescence dequenching signal of the lipophilic dye, DiD, near 400 s indicates viral fusion. **(c)** The trajectory of a Cy3/CypHer5-labeled virus inside a cell. Color coding of the trajectory is similar to that in **(b)**. The green star indicates the initial acidification site (to pH ~6). **(d)** Time trajectories of the velocity (black) and the fluorescent emission ratio of CypHer5 (a pH-dependent dye) and Cy3 (a pH-independent dye) (red) of the virus. The pH scale is labeled according to calibration measurements performed on virus particles *in vitro*. Scale bars: 10 μ m. (Figure adopted from Ref.[28])

Figure 6.