

Differential Requirements of Rab5 and Rab7 for Endocytosis of Influenza and Other Enveloped Viruses

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Enveloped viruses often enter cells via endocytosis; however, specific endocytic trafficking pathway(s) for many viruses have not been determined. Here we demonstrate, through the use of dominant-negative Rab5 and Rab7, that influenza virus (Influenza A/WSN/33 (H1N1) and A/X-31 (H3N2)) requires both early and late endosomes for entry and subsequent infection in HeLa cells. Time-course experiments, monitoring viral ribonucleoprotein colocalization with endosomal markers, indicated that influenza exhibits a conventional endocytic uptake pattern – reaching early endosomes after approximately 10 min, and late endosomes after 40 min. Detection with conformation-specific hemagglutinin antibodies indicated that hemagglutinin did not reach a fusion-competent form until the virus had trafficked beyond early endosomes. We also examined two other enveloped viruses that are also pH-dependent for entry – Semliki Forest virus and vesicular stomatitis virus. In contrast to influenza virus, infection with both Semliki Forest virus and vesicular stomatitis virus was inhibited only by the expression of dominant negative Rab5 and not by dominant negative Rab7, indicating an independence of late endosome function for infection by these viruses. As a whole, these data provide a definitive characterization of influenza virus endocytic trafficking and show differential requirements for endocytic trafficking between pH-dependent enveloped viruses.

Key words: endosomal trafficking, influenza virus, Rab5, Rab7, SFV, VSV

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Many viruses use endocytosis as a route of entry into host cells (1,2). Following binding to cell surface receptors, the virus is internalized into the endocytic network in a manner that is generally considered to be clathrin dependent. In many cases endocytosis is crucial for virus infection, as the low-pH environment of the endosome triggers membrane fusion events crucial for genome release (3). As well as allowing the acidification often required by viruses for fusion and/or uncoating, endosomes can be exploited and used for sorting and transit through the cytoplasm. Whereas the role of endosome acidification during virus entry is clear in many cases, much less is known about

potential sorting and trafficking roles of endosomes for viral infection.

Influenza virus is known to enter cells by endocytosis through both clathrin- and nonclathrin-mediated pathways (4,5). The virus has been well established to require the progressive pH drop of the endocytic pathway for infection. Endosomal acidification is essential for two distinct events in influenza virus entry. First, the low pH triggers a conformational change in the hemagglutinin (HA) viral glycoprotein to expose a fusion peptide (6,7). Fusion of the virus envelope to the endosomal membrane via HA occurs at a defined pH, which can vary for different virus strains (8). Typically, a pH of approximately 5.5 is required (9,10). Based on this requirement for low pH, it was originally proposed that virus entered cells via lysosomes (4,11). Subsequently, the site of entry was determined to be endosomes (10); however, the actual identity of the functional compartment for virus fusion has not yet been identified. A second low-pH step is required for uncoating of the influenza vRNPs, as the interior of the virus becomes acidified via the M2 ion channel (12,13). For uncoating, a less stringent pH requirement of approximately 6.0 seems to be sufficient (14). Once released into the cytoplasm, the viral ribonucleoproteins (vRNPs) are then transported into the nucleus, by way of nuclear pore complexes, for replication (15). For the initial steps in the influenza virus life cycle, endosomal trafficking is essential in order to deposit the virus in a low-pH compartment.

Other enveloped viruses also enter host cells by endocytosis in a pH-dependent manner, including Semliki Forest virus (SFV) and vesicular stomatitis virus (VSV). It has been well established that SFV requires clathrin-mediated endocytosis for productive entry (5,16) and is known to undergo fusion at a pH of approximately 6.2 – roughly the environment of early endosomes (2,3,17,18). VSV, although also well established to enter cells through a pH-dependent mechanism and endocytosis, is thought to have a wider pH range for fusion of roughly 5.5–6.3 – or the stretch of the endocytic pathway from early to late endosomes (3,17,19,20). Therefore, like influenza virus, the exact sites of fusion for SFV and VSV are as yet unknown.

The endocytic pathway that the above viruses must traverse to cause a successful infection consists of temporally and physiologically distinct endosome populations (21). Early endosomes, which serve as sorting vesicles for incoming ligands, are usually reached by cellular ligands, such as epidermal growth factor (EGF) and transferrin, at

approximately 5–15 min after binding to the cell surface. Early endosomes can progress to recycling endosomes, which deliver endocytosed material back to the cell surface, or to late endosomes and lysosomes. Late endosomes, or multivesicular bodies, are usually reached by ligands at later times after penetrating the plasma membrane of the cell, and have a significantly lowered pH compared to early endosomes (22).

The trafficking within, and fusion of, endosomes is controlled by Rab guanosine triphosphatases (GTPases), which act as molecular switches that target the movement of endocytic vesicles, and are also involved in vesicle formation (23–25). It is believed that Rabs determine endocytic vesicle specificity by their localization and by their recruitment of effector proteins unique to each endosome population (26), e.g. Rab5 regulates the function of early endosomes (27), while Rab7 regulates late endosomes (28,29).

The expression of dominant-negative mutants of Ras-related GTPases is a very specific and powerful tool for

understanding their physiological role (30). For example, a dominant-negative form of Rab5, such as Rab5 S34N, is unable to interact with EEA1 (early endosomal autoantigen 1) and the expression of such mutants of Rab5 is well established to disable early endosome function (31). Likewise, expression of dominant-negative mutants of Rab7, such as Rab7 T22N, is well known to inhibit late endosome function (29,32).

The use of dominant-negative mutant Rab proteins to examine viral endocytic trafficking has not been routinely performed. However, it has been shown that adenovirus requires functional early endosomes for entry and infection based on the use of a dominant-negative Rab5 mutant (33). Here, through the use of Rab5 and Rab7 dominant-negative mutants, we demonstrate that influenza virus requires functional early and late endosomes for successful trafficking and subsequent infection. Through colocalization of vRNPs and endosomal markers, we show that influenza virus is present in early and late endosomes in a temporally conventional endocytic uptake. Additionally, we

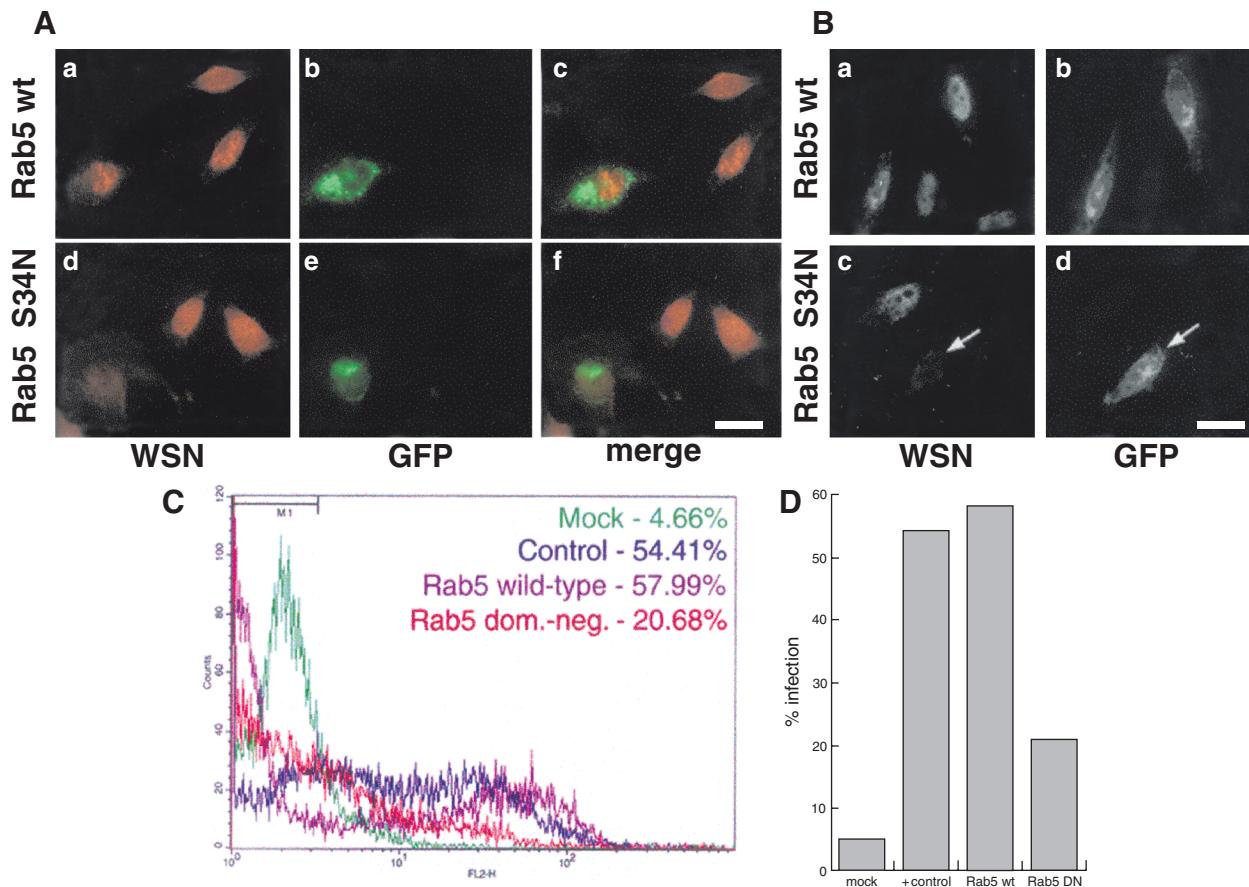


Figure 1: Expression of dominant-negative Rab5 prevents influenza infection. HeLa cells were transiently transfected with either wild-type or dominant-negative Rab5 GFP-tagged constructs before infection. Influenza infection was performed as either (A) an entry assay (100–200 p.f.u./cell for 1 h) or (B) a 4-h infection (1–5 p.f.u./cell). Influenza infection was monitored by indirect immunofluorescence. Scale bars = 8 µm. (C) and (D) After transfection, cells were infected with 1–5 p.f.u./cell influenza virus for 2 h. Infection was monitored by FACS analysis. In all cases, influenza virus was detected by the monoclonal antibody against NP. Graph data represents the percentage of infected cells as determined by FACS analysis. The M1 gate designates the population of uninfected cells.

describe a requirement of SFV and VSV for functional early, but not late, endosomes. Together, these data comprise a model for the cellular mechanisms of influenza virus trafficking in comparison to other enveloped viruses.

Results

Expression of dominant-negative Rab5 prevents influenza virus infection

It has been hypothesized that influenza virus must traffic through both early and late endosomes to reach a compartment with a pH low enough for viral fusion out of the endocytic pathway (9,10). We first tested the requirements for early endosome trafficking during influenza virus entry. We examined influenza virus infection in cells expressing dominant-negative Rab5 (S43N). In a virus entry assay, monitored by indirect immunofluorescence, cells expressing dominant-negative Rab5 showed no detectable influenza vRNP signal in the nucleus, indicative of a block in virus infection (Figure 1A, panel d, arrow).

vRNP signal was also absent from the cytoplasm as the virus, unable to reach a low-pH compartment, has not yet uncoated for the NP protein to be available to antibody. The same result was obtained in a 4-h infection at a lower multiplicity of infection (M.O.I.), with again no detectable virus replication in the nucleus of Rab5 S34N-expressing cells (Figure 1B, panel c, arrow). In both cases, the overexpression of wild-type Rab5 appeared to have no effect on influenza infection (panels a and b), as shown by strong labeling of vRNPs in the nucleus.

We next used fluorescence activated cell sorting (FACS) analysis in order to quantitate our immunofluorescence microscopy results. Compared to a control infection (untransfected cells) rate of 54%, the infection in dominant-negative Rab5-expressing cells dropped to 21% (Figure 1C,D). Taking into account our transfection efficiency of approximately 70%, it is likely that these 21% comprise an untransfected cell population. Overexpression of wild-type Rab5 caused a slight increase in the infection rate to 58%. These data indicate that influenza virus requires a Rab5-dependent step for entry, strongly suggesting that the virus must traffic

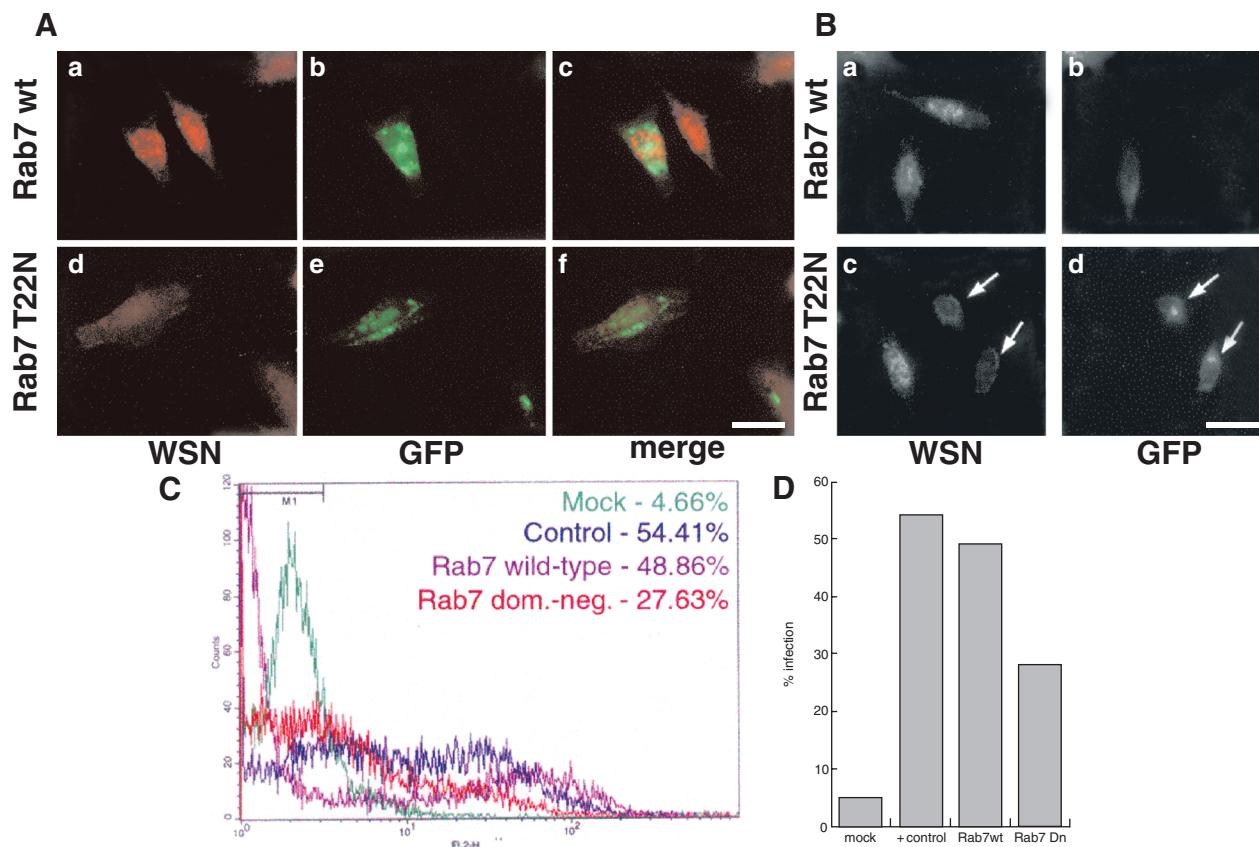
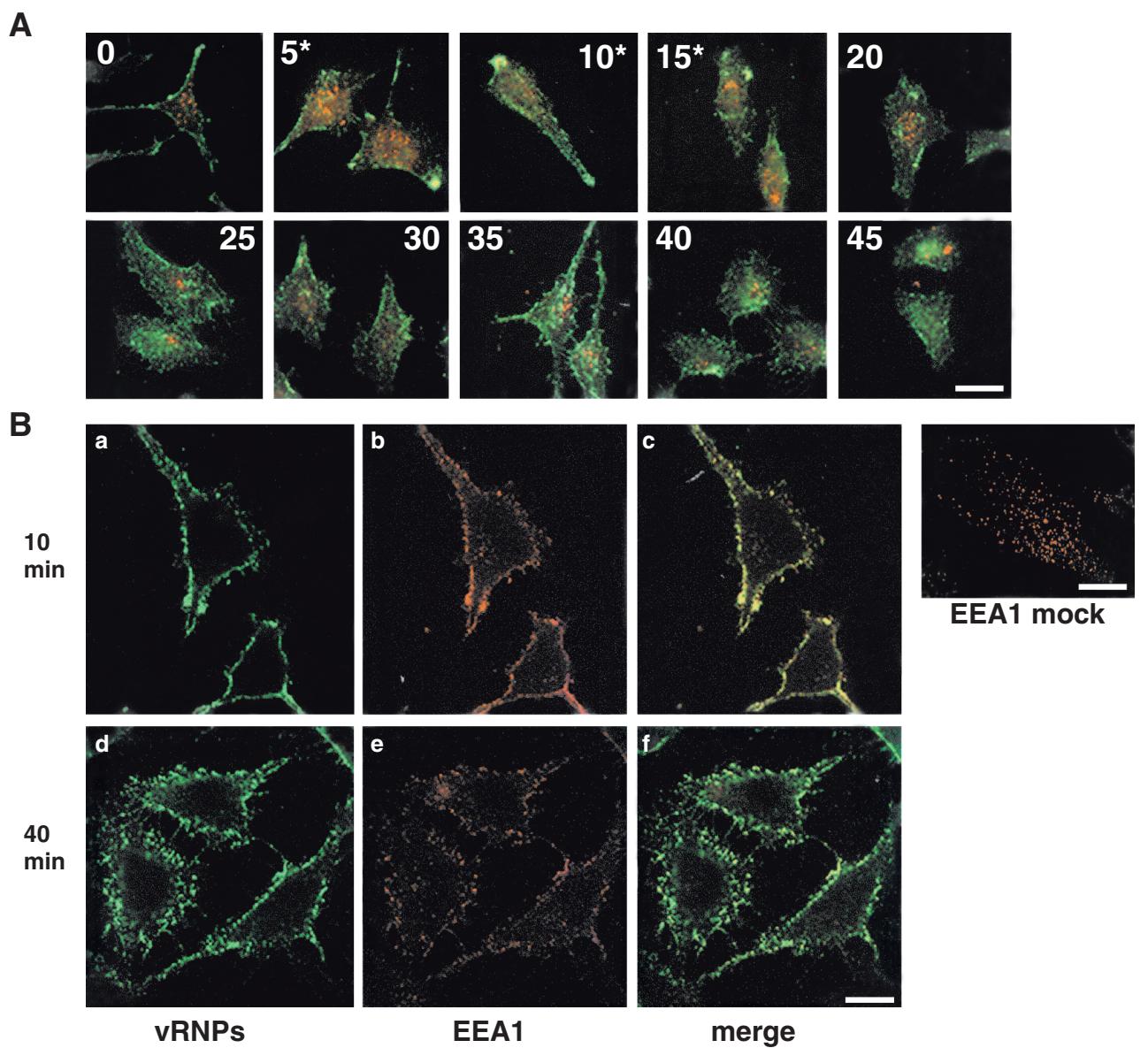


Figure 2: Expression of dominant-negative Rab7 prevents influenza infection. HeLa cells were transiently transfected with either wild-type or dominant-negative Rab7 GFP-tagged constructs before infection. Influenza infection was performed as either (A) an entry assay (100–200 p.f.u./cell for 1 h) or (B) a 4-h infection (1–5 p.f.u./cell). Influenza infection was monitored by indirect immunofluorescence. Scale bars = 8 µm. (C) and (D) After transfection, cells were infected with 1–5 p.f.u./cell influenza virus for 2 h. Infection was monitored by FACS analysis. In all cases, influenza virus was detected by the monoclonal antibody against NP. Graph data represents the percentage of infected cells as determined by FACS analysis. The M1 gate designates the population of uninfected cells.



through the early endosome for successful infection. The inhibition due to the expression of Rab5 T34N most likely reflects a defect in early endosome function, but an inhibition of endocytosis cannot be ruled out, as yet.

Expression of dominant-negative Rab7 prevents influenza virus infection

It is thought that the late endosome can provide the correct pH (approximately 5.5) for influenza virus fusion out of the endocytic pathway (9,10). To test the requirement for functional late endosome trafficking in influenza virus infection, we examined the infection of the virus in cells expressing dominant-negative Rab7 (T22N). By indirect immunofluorescence microscopy, a virus entry assay demonstrated that expression of dominant-negative Rab7

prevented influenza virus infection, as demonstrated by a lack of vRNP nuclear localization (Figure 2A, panel d). The same result was obtained after a 4-h infection (Figure 2B, panels c and d). In contrast to expression of Rab5 S34N, some vRNP signal was apparent in internal vesicles with Rab7 T22N expression. As with Rab5, overexpression of wild-type Rab7 appeared to have no effect on influenza virus entry, with strong vRNP localization to the nucleus (Figure 2A,B, panels a and b). FACS analysis indicated an infection rate of 28%, down from 54% in control infected cells (Figure 2C,D). Again, taking into account that approximately 30% of the cell population remains untransfected, it is likely that there is a complete block of infection in transfected cells. Infection in cells expressing wild-type Rab7 was similar to that of control cells. These data indicate that influenza virus requires functional late

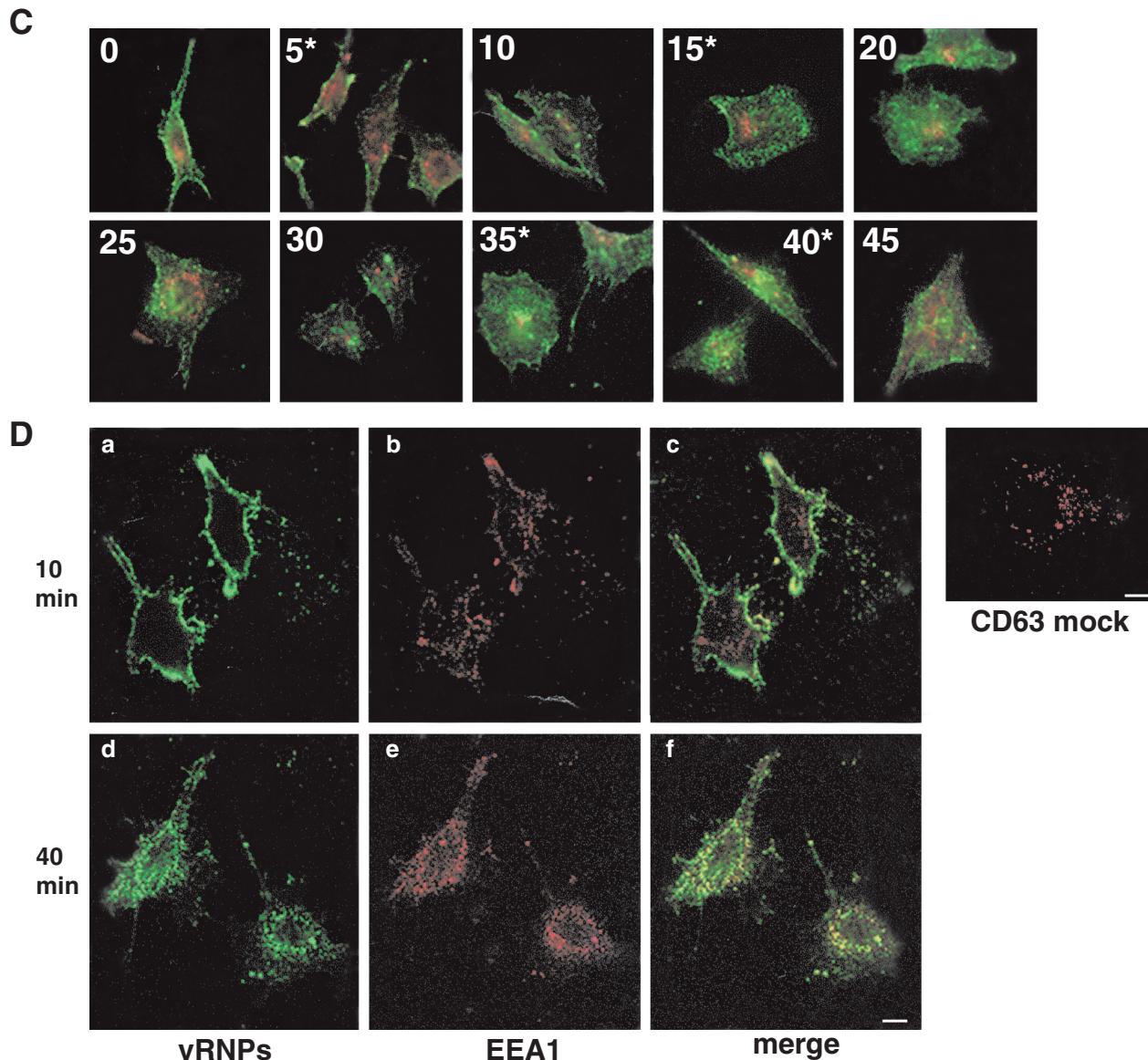


Figure 3: Influenza virus colocalizes with endosomal markers at distinct time points. HeLa cells were infected with influenza virus at an M.O.I. of 100–200 p.f.u./cell. Virus was bound to cells at 4 °C for 90 min. Cells were then shifted to 37 °C for indicated time points. (A) Indirect immunofluorescence microscopy with early endosome marker. (B) Confocal microscopy with early endosome marker. (C) Indirect immunofluorescence microscopy with late endosome marker. (D) Confocal microscopy using late endosome marker. Influenza virus was detected by the monoclonal antibody against NP. EEA1 was used as a marker of early endosomes; CD63/Lamp3 as a marker of late endosomes. Scale bars = 8 μm.

endosome trafficking for infection, suggesting the virus must travel at least this far in the endocytic pathway for fusion to occur.

Influenza virus traffics through early and late endosomes and acquires a low-pH form at distinct time points during infection

To confirm the requirement of both early and late endosomes for influenza virus infection, and to determine the timing of virus trafficking through the endosomal network,

an infection time-course was performed to analyze the colocalization of influenza vRNPs with endosomal markers. Early endosomal autoantigen 1 (EEA1) served as a marker for early endosomes (34). Based on immunofluorescence microscopy, influenza vRNPs appeared to be colocalizing with EEA1 at approximately 5–15 min post infection (Figure 3A). This was confirmed by confocal microscopy. At a 10-min time point, most of the vRNP signal colocalizes with EEA1 near the periphery of the cell (Figure 3B, panel c). Interestingly, the EEA1 distribution was much more

peripheral than that seen with mock-infected HeLa cells. At a 40-min time-point, the majority of this colocalization had disappeared (panel f), the remaining signal likely being slow-moving virus. CD63/Lamp3 was used as a marker for late endosomes (35). The majority of influenza vRNPs appeared to be colocalizing with CD63 at approximately 35–45 min post infection (Figure 3C). This was confirmed by confocal microscopy. At a 10-min time-point, there was no colocalization between the vRNPs and CD63 (Figure 3D, panel c); however, at 40 min the vRNPs colocalized strongly with the late endosome marker in the perinuclear region of the cell (panel f). The yellow signal at the periphery of the cells is likely due to accidental overlap of signal in areas of strong labeling, rather than true colocalization. Overall, these data support the results obtained with the dominant-negative Rab constructs – that influenza virus transits through both early and late endosomes during its entry into host cells. Influenza virus appears to undergo this trafficking by the conventional endocytic route, concentrating in early endosomes at around 5–15 min and late endosomes at about 35–45 min post infection. By 60 min, the virus had been released from the endocytic pathway for transport into the nucleus for replication (data not shown).

Influenza virus entry into host cells is known to be pH dependent. To correlate our colocalization data with the pH requirements for fusion, we employed conformation-specific antibodies against the HA protein (A2 and N2). These antibodies detect HA in its neutral pH conformation (N2; above pH 6.0) and in its acidified form with the fusion peptide exposed (A2; below pH 6.0) (36). In cells infected

with influenza virus for 10 min, the time needed for the virus to concentrate in early endosomes, principally the neutral form of HA was detectable (Figure 4, panels a and c). However, after 40 min of infection, the viral HA was primarily in its acidified conformation, as judged by A2 antibody-reactivity (panels b and d). By this time-point, the virus has reached late endosomes with a lowered pH. These data indicate, *in vivo*, that the influenza virus HA glycoprotein alters its conformation as it traffics from early to late endosomes due to the pH drop between the endosome populations.

Dominant-negative Rab5, but not Rab7, expression prevents Semliki Forest virus (SFV) infection

We next examined the effects of Rab5 and Rab7 on the entry of two other enveloped viruses, SFV and VSV. The spike (E1) protein of Semliki Forest virus (SFV) is known to be activated at a pH of 6.2 (2,18) – roughly the environment of early endosomes. To determine the endosomal trafficking requirements of SFV, cells were transfected with the wild-type and dominant-negative Rab5 and Rab7 plasmids before infection. Transfection with either wild-type Rab5 or Rab7 had no effect on SFV infection (Figure 5, panels a). However, transfection with dominant-negative Rab5 resulted in a block on virus SFV entry and infection, indicating a requirement for a Rab5-mediated event, likely that of a functional early endosome (Figure 5A, panel e). In contrast, transfection with dominant-negative Rab7 had no effect on infection (Figure 5B, panel e). These data indicate that SFV requires trafficking in early, but not late, endosomes. This further suggests that SFV fuses out of

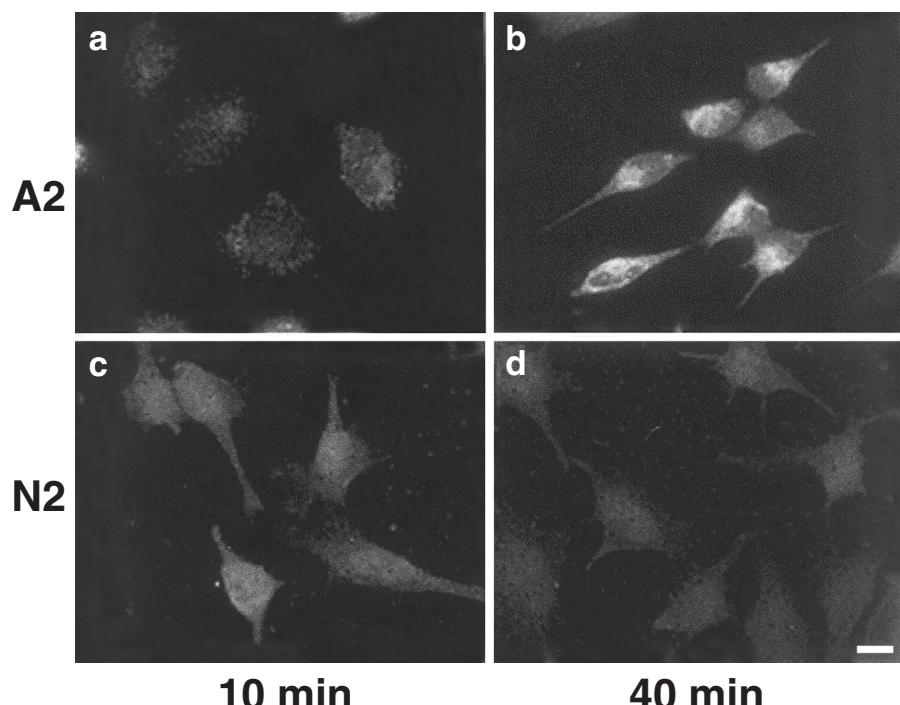


Figure 4: Influenza virus HA conformational change occurs late in endocytic pathway. HeLa cells were infected with the A/X-31 strain (H3N2) of influenza virus at an M.O.I. of 100–200 p.f.u./cell for 10 or 40 min. Cells were examined by indirect immunofluorescence microscopy. Infection was detected by the conformation-specific monoclonal antibodies against HA (N2 = neutral conformation; A2 = acidified conformation). Scale bars = 8 μm.

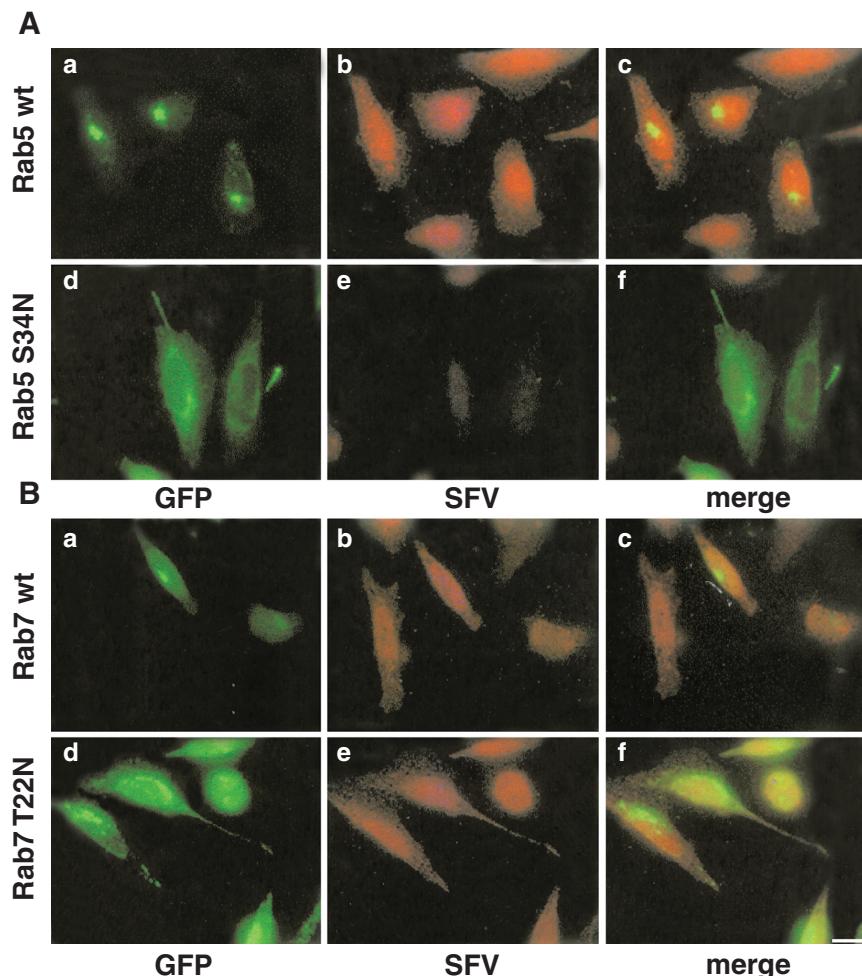


Figure 5: Expression of dominant-negative Rab5, but not Rab7, prevents SFV infection. HeLa cells were transiently transfected with either wild-type or dominant-negative (A) Rab5 or (B) Rab7 GFP-tagged constructs before infection. Cells were infected with SFV at 1–5 p.f.u./cell for 5 h. Infection was monitored by indirect immunofluorescence assay using the monoclonal antibody against E1-1. Scale bars = 8 μm.

the endocytic pathway from a vesicle earlier than late endosomes.

Dominant-negative Rab5, but not Rab7, expression prevents vesicular stomatitis virus (VSV) infection

We also examined the role of Rab5 and Rab7 for VSV, a virus where the pH requirement for fusion is less clear than for SFV and influenza virus. VSV appears to have a wider range of pH that will allow for fusion, approximately 5.5–6.2 (2,37). To examine the endocytic trafficking of VSV, cells expressing the Rab constructs were infected. Again, expression of wild-type Rab5 or Rab7 appeared to have no effect on virus infection (Figure 6, panels a). Cells transfected with dominant-negative Rab5 were unable to be infected by VSV, indicating a lack of a Rab5-mediated event, again likely the lack of a functional early endosome, prevents virus entry (Figure 6A, panel e). Expression of dominant-negative Rab7 appeared to have no effect on VSV infection (Figure 6B, panel e). These data indicate that VSV requires early endosomal, but not late endosomal, trafficking for entry and subsequent

infection. This suggests that VSV is capable of fusing out of the endocytic pathway at the level of early endosomes.

Discussion

Through the expression of dominant-negative Rab constructs, we have shown that influenza virus requires trafficking in both early and late endosomes for entry into and subsequent infection of host cells. These data are in agreement with previous work, which indicates influenza virus requires low pH for release from the endocytic pathway (9,10). Colocalization studies using defined endosomal markers supported our results from the Rab mutants, confirming the influenza virus is present in early and late endosomes at distinct times during infection. Additionally, analysis with HA conformation-specific antibodies suggests that the pH-mediated release of the HA fusion peptide occurs late in the endocytic pathway. The entry of SFV and VSV, on the other hand, appears to

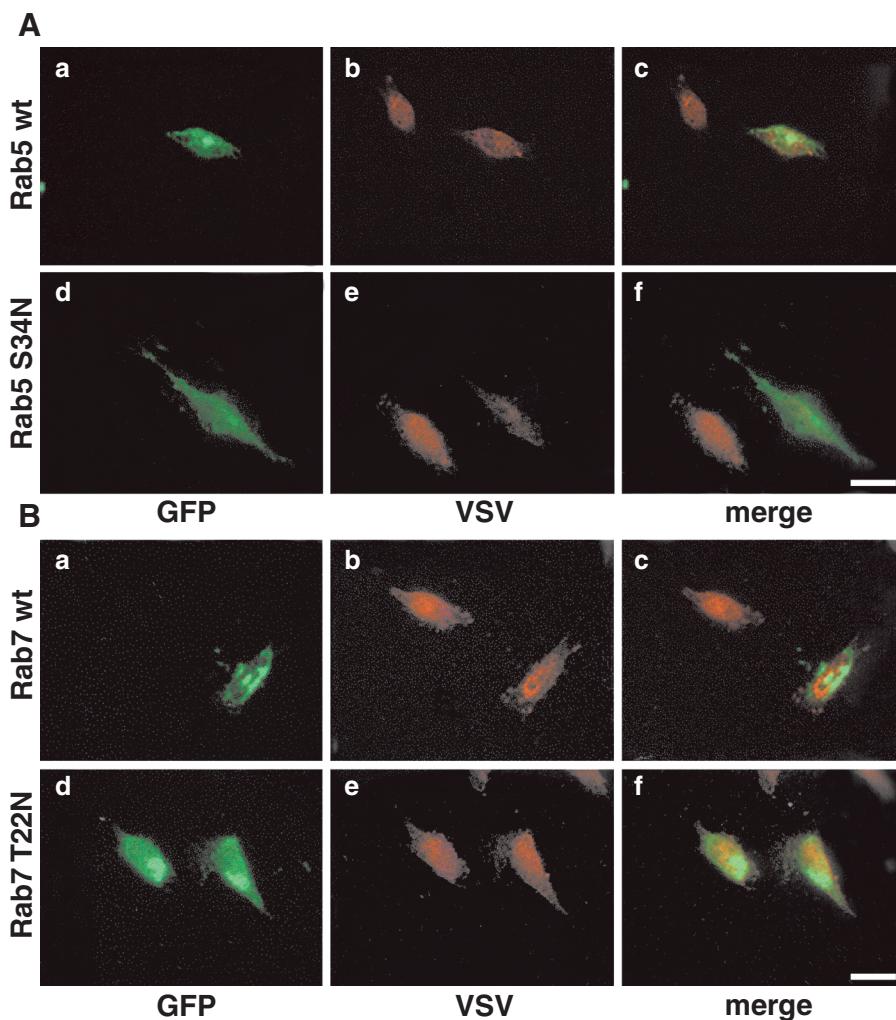


Figure 6: Expression of dominant-negative Rab5, but not Rab7, prevents VSV infection.
HeLa cells were transiently transfected with either wild-type or dominant-negative (A) Rab5 or (B) Rab7 GFP-tagged constructs before infection. Cells were infected with VSV at 1–5 p.f.u./cell for 6 h. Infection was monitored by indirect immunofluorescence assay using the monoclonal antibody against the viral G glycoprotein. Scale bars = 8 μ m.

require only early endosome trafficking, suggesting fusion at an earlier step of the endocytic pathway.

Our results with the dominant-negative Rab mutants give support to the hypothesis that influenza virus traffics through both early and late endosomes as it enters host cells by endocytosis. In contrast, both VSV and SFV appear to require trafficking only through early endosomes. These results agree with the demonstrated fusion capabilities of each viral glycoprotein. The spike glycoprotein of SFV is known to undergo fusion at a pH of 6.2 (18). The independence of SFV on a functional late endosome is evidence that the virus can fuse out of the endocytic pathway at an early stage. Previous studies on SFV fusion involving the control and monitoring of endosomal acidification also suggested the virus fuses from the early endocytic pathway at a relatively high pH (38,39). The same appears to be true for VSV, which has been described as having a pH range of 5.6–6.3 for G protein fusion (17,19). The specific strain used here, VSV tsO45, has been determined to have a pH fusion point of 6.3 (M. Whitt, personal communication). Despite the pH range, VSV appears

capable of fusing from a point early on in the endocytic pathway, and late endosomes do not appear to be necessary for infection. The HA glycoprotein of influenza virus has been described to undergo fusion at a pH of 5.5 (9,10). The dependence of influenza virus on functional early and late endosomes suggests that the virus must be able to reach bona fide late endosomes for fusion and release from the endocytic pathway. Analysis with conformation-specific antibodies against HA indicates that the fusion peptide is not exposed until late in entry, approximately at the time when virus is concentrated in late endosomes.

One feature of our results was a slight, but significant, increase in influenza virus infection with the overexpression of wild-type Rab5 (see Figure 1D). This type of increased virus entry has been observed previously with adenovirus (33), where the higher rate of infection was attributed to an increase in number and size of early endosomes, induced by the over-expression of Rab5. It is possible that a similar effect is occurring for influenza virus. We did not observe a similar increase in the rate of

influenza infection upon expression of wild-type Rab7. There are several possible explanations for this finding. It may be that this slight effect is discernible only at the commencement of endocytic trafficking, when ligands are highly concentrated. This may be a phenomenon unique to the early/sorting endosome itself. Or it may be that the late endosome/Rab7 compartment is not rate limiting for infection.

There is only one current example where Rab proteins have been used to look at virus entry. In the case of adenovirus, it was found that the rate of virus entry increased upon over-expressing wild-type Rab5 and decreased with the expression of dominant-negative Rab5, indicating a role for early endosome trafficking for adenovirus infection (33). Our results for influenza, SFV and VSV displayed a similar infection pattern in Rab5-expressing cells. Adenovirus may itself be capable of stimulating its own uptake into early endosomes through a signaling cascade, as it has been shown that virus binding to its α_v integrin receptor can activate phosphatidylinositol 3-kinase [PI(3)-kinase] (40). However, it should be noted that this effect is not specific to the virus, but rather to the specific cellular integrin receptor to which the virus binds. Furthermore, it is known that PI(3)-kinase is an activator of Rab5 and serves to regulate the rate of endocytosis (41). It is as yet unclear if influenza virus would have similar signal activation abilities; however, based on our time-course studies, influenza virus does not appear to affect the conventional uptake of ligands via endocytosis.

While we saw no apparent effect of influenza virus on the kinetics of endocytosis, one observation that we routinely made was a change in the distribution of both early and late endosomes upon influenza virus infection. This was most apparent for early endosomes (see Figure 3). In mock-infected HeLa cells, the localization of EEA1-positive early endosomes was scattered throughout the cytoplasm. However, upon influenza virus entry for 10 min, the EEA1 staining pattern showed early endosomes highly concentrated around the periphery of the cell, in the vicinity of the plasma membrane. By 40 min, a peripheral distribution was still present, but was less pronounced. The reasons for this relocalization of endosomes are presently unclear, but we would speculate that the virus actively recruits early endosomes to the site(s) of infection. The mechanism for such recruitment is not known, but is in line with reports of increased fluid phase endocytosis in influenza-infected human airway epithelia at early time-points of infection (42). It is also reminiscent of parasites that recruit endocytic compartments to their site of entry (2). In addition to the above microscopic analysis, studies involving early/late endosome fractionation from infected cells at 10- and 40-min time-points are currently being attempted to further confirm movement through these endosome populations.

We have recently demonstrated that influenza virus can infect cells in the absence of clathrin-mediated endocyto-

sis (5). To determine the endosomal trafficking of virus particles entering by nonclathrin-mediated endocytosis, cells transiently transfected with GFP-Eps15 Δ 95-295 were analyzed after an infection time-course, with no obvious differences in the kinetics of infection or the colocalization with EEA1 and CD63 (data not shown). These data indicate that when entering by an alternative nonclathrin-mediated endocytic route, the influenza virus is still trafficked through the expected endosome populations. Our data for influenza that show a specific requirement for late endosome/lysosome-targeted trafficking that may relate specifically to the relative roles of clathrin and nonclathrin pathways for internalization for this virus vs. SFV or VSV. It has been shown recently that elimination of clathrin in cells has no apparent effect on lysosome biogenesis (43). It is possible that viruses can selectively use clathrin-independent endocytosis if they have a requirement for entry into the lysosome-targeted pathway of endocytic traffic.

We used HeLa cells for our experiments as they are very efficiently transfected and allow meaningful quantitation of infection levels in the presence of transiently transfected protein. These cells are not routinely used for infection of influenza virus, SFV or VSV. However, apart from differences in both relative infection efficiencies and infectious virus release from HeLa cells, we observed no significant differences in the expression patterns of any of the viral proteins tested between HeLa and other cells, such as MDCK or BHK. In general, we do find that the kinetics of endocytosis are somewhat slower in HeLa cells (for both viruses and cellular ligands), and this may explain the differences in our entry kinetics (40 min to the site of fusion) with those of others (10 min to the site of fusion in MDCK cells) (10). In addition to the cell type used for experiments, the viral strain may also affect endocytic trafficking pathways. While the two influenza virus strains used for these experiments, A/WSN/33 (H1N1) and A/X-31 (H3N2), were trafficked to late endosomes and appeared to require the low-pH environment of the late endosome for fusion, this trafficking pattern may vary for viral strain capable of fusion at a higher pH. There are viral fusion mutants of the X-31 strain available in which HA has been described to fuse at 1.2 pH units above wild-type (44). It is possible that such fusion mutants would be unaffected by the expression of dominant-negative Rab7, not requiring trafficking through late endosomes as they may fuse from early endosomes.

Overall, our data give credence to the hypothesis that influenza virus requires trafficking through both early and late endosomes. They also support the requirement of influenza virus to reach a sufficiently low-pH compartment (approximately 5.5) for fusion out of the endocytic pathway. VSV and SFV, which require a smaller drop in pH to trigger fusion, do not need to undergo such extensive endocytic trafficking and can infect cells via the early endosome. In general, the use of dominant-negative Rab

proteins and colocalization studies provide a clear means to determine the function of the endocytic pathways during virus entry.

Materials and Methods

Cells, viruses and infections

HeLa cells (American Type Culture Collection, Rockville MD, USA) were maintained in α-MEM containing 10% calf serum, 100 U/ml penicillin and 10 µg/ml streptomycin and passaged twice weekly. Influenza A/WSN/33 (H1N1) and A/X-31 (H3N2) stocks were grown in MDBK or MDCK cells, or in 10-day-old embryonated eggs, and plaque titered on MDCK cells (45). Unless stated otherwise in the figure legend, the A/WSN/33 strain was used for all experiments. Semliki Forest virus (SFV) strain M1 was provided by Dr Margaret Kielian, Albert Einstein College of Medicine. VSV (strain tsO45) was obtained from American Type Culture Collection (Rockville, MD, USA). Infections were performed essentially as described previously (46). Briefly, viral stocks were diluted in RPMI 1680 medium containing 0.2% BSA and buffered to pH 6.8 with HEPES. Virus was adsorbed for 90 min at 4 °C, cells were then maintained in growth medium containing 2% serum at 37 °C for either 60 min (at ~100–200 p.f.u. per cell), or at ~1–5 p.f.u. per cell. At the lower M.O.I., influenza virus was allowed to infect for 4 h, SFV for 5 h and VSV for 6 h.

Transfections

Constructs of wild-type GFP-tagged human Rab5A and human Rab7, as well as dominant-negative mutants (Rab5 S34N and Rab7 T22N), cloned as N-terminal GFP fusions in the pGreenLantern vector (Gibco-BRL, Grand Island NY, USA), were provided by Dr Craig Roy, Yale University. Transfections were performed using the Effectene transfection kit (Qiagen, Valencia CA, USA) according to manufacturer's protocols. For transfection, HeLa cells were grown on coverslips in 24-well plates and transfected with 1.0 µg DNA. Transfections were typically allowed to proceed for 16–18 h before infection or ligand uptake. On average, we obtained >65% transfection efficiency of these plasmids in our cell culture system.

Indirect immunofluorescence microscopy

Preparation of cells for indirect immunofluorescence microscopy was performed as described previously (47). Influenza virus nucleoprotein (NP) was detected using the monoclonal antibody H10, L16–4R5 (American Type Culture Collection, Rockville MD, USA). Semliki Forest virus was detected with the E1-1 monoclonal antibody (provided by Dr Margaret Kielian, Albert Einstein College of Medicine). VSV was detected with the G protein monoclonal antibody P5D4 (provided by Dr Ira Mellman, Yale University). Expression of Rab constructs was detected by direct GFP fluorescence. Early and late endosomes were detected by the monoclonal antibodies against early endo-

some autoantigen 1 (EEA1) (Transduction Laboratories, Palo Alto CA, USA) and CD63/Lamp3 (Chemicon, Temecula CA, USA), respectively. EGF receptor was detected using an anti-EGFR monoclonal antibody (Calbiochem, San Diego CA, USA). Secondary antibodies used were Alexa 488-labeled (green) or Alexa 568-labeled (red) goat anti-mouse IgG (Molecular Probes). Cells were viewed using a Zeiss Axioskop 2 plus microscope with a 63X objective lens. Images were captured with a Zeiss AxioCam using Axiovision 3.0.6.1 software (Carl Zeiss, Thornwood NY, USA) before being transferred into Adobe Photoshop (Adobe Systems, San Jose CA, USA). Confocal microscopy was performed using an Olympus Fluoview confocal station. Alexa 488 was excited with the 488 nm line of an Argon laser and Alexa 568 was excited with the 568 nm line of a Krypton laser. Cells were viewed with a 60× objective lens and images were captured with Fluoview software (Olympus, Melville NY, USA) before being transferred into Adobe Photoshop.

FACS analysis

For flow cytometry preparation, cells were trypsinized, washed in PBS, fixed in 3% paraformaldehyde/PBS and permeabilized in 0.075% saponin in 10% goat serum/PBS. Cells were incubated with the monoclonal antibody to influenza NP for 30 min, followed by Alexa 488-labeled or Alexa 568-labeled goat anti-mouse IgG for 30 min. Cells were analyzed on a FACSCalibur cytometer using CellQuest 3.1f software (Becton Dickinson Immunocytometry Systems, Plao Alto CA, USA). At least 10 000 cells were analyzed for each sample.

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