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The clathrin endocytic pathway in viral infection

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How important is the clathrin-dependent endocytic pathway for entry of viruses into host cells? While it is widely accepted that Semliki Forest virus (SFV), an enveloped virus, requires this pathway there are conflicting data concerning the closely related Sindbis virus, as well as varying results with picornaviruses such as human rhinovirus 14 (HRV 14) and poliovirus. We have examined the entry mode of SFV, Sindbis virus, HRV 14 and poliovirus using a method that identifies single infected cells. This assay takes advantage of the observation that the clathrin-dependent endocytic pathway is specifically and potently arrested by overexpression of dynamin mutants that prevent clathrin-coated pit budding. Using HeLa cells and conditions of low multiplicity of infection to favor use of the most avid pathway of cell entry, it was found that SFV, Sindbis virus and HRV 14 require an active clathrin-dependent endocytic pathway for successful infection. In marked contrast, infection of HeLa cells by poliovirus did not appear to require the clathrin pathway.

Keywords: clathrin adaptors/coated vesicles/endocytosis/viral entry

Introduction

Initiation of a viral infection requires entry of the virus into the host cell. The viral genome can enter the cytoplasm directly at the cell surface, by penetrating the plasma membrane, or after endocytosis by penetrating membranes of intracellular organelles such as the endosome.

In the past, a standard method for assessing the route of viral entry has been to use direct electron microscopic observation of cells at early time points after infection. Because this approach requires a very high multiplicity of infection (MOI) of virus to ensure that images of virus particles can be found, it suffers from a tendency to visualize viruses bound to non-productive sites as well as to their specific cellular target locations. It has been difficult to resolve contradictions between different studies using electron microscopy. For example, in a study of poliovirus—a non-enveloped picornavirus—virus particles were found in association with pits (presumably clathrin-coated) and in completely invaginated coated vesicles (Zeichhardt *et al*., 1985; Willingmann *et al*., 1989). In another study, however, no viruses were found surrounded by cellular membranes, and viral particles were observed only on the surface of the plasma membrane (Dunnebacke *et al*., 1969). Similarly, Semliki Forest virus (SFV), an enveloped alphavirus of the togavirus family, has been reported to enter into the endocytic compartment following its uptake from the cell surface through clathrin-coated pits (Helenius *et al*., 1980, 1985; White *et al*., 1980; Marsh *et al*., 1984). In another study, however, SFV was also found inside non-coated pits and vesicles, leading to the proposal that entry via the clathrin pathway is fortuitous (Hase *et al*., 1989).

An alternative way to study the site at which a viral genome enters host cells is to use pharmacological agents or cellular conditions that block the acidification of the endosomal and lysosomal compartments. This approach also has its problems. For example, some of the studies performed on poliovirus appeared to show that exposure to a low-pH intracellular compartment is required for successful infection (Madshus *et al*., 1984), whereas others suggested that this is not the case (Perez and Carrasco, 1993). Results with enveloped viruses have been clearer, and there is general agreement that SFV, the related Sindbis virus and influenza virus, among others, require exposure to an acidic intracellular compartment for infection (Helenius *et al*., 1982; Marsh *et al*., 1982; Talbot and Vance, 1982; Perez and Carrasco, 1993).

One possible way to access an acidic compartment is to enter the cell through the clathrin-mediated pathway, by 'hitching a ride' with a protein(s) targeted to the endosome. The only direct attempt so far to examine the question of whether a virus enters its host cell through the clathrin-mediated pathway used SFV (Doxsey *et al*., 1987). In this case, the introduction of anti-clathrin antibodies into the cytosol of host cells blocked infection by ~60%. However, as the authors pointed out, the fact that this blockage is only partial must mean either that the antibodies do not inhibit the clathrin pathway completely, or that the virus can also enter through an alternative pathway that does not require clathrin.

In this report, we describe results using a new method that allows us to determine unambiguously whether a virus uses clathrin-coated pits and vesicles to enter into cells, taking advantage of the observation that the formation of clathrin-coated vesicles requires dynamin (van der Bliek *et al*., 1993). Dynamin is a protein of ~100 kDa that facilitates the budding of clathrin-coated pits, leading to the formation of coated vesicles, in a GTP-dependent manner. The expression of dominant-negative dynamin mutants that either fail to load GTP (dynamin^{K44A}) or fail to hydrolyze GTP at the non-permissive temperature (dynamin^{ts}), specifically blocks the formation of clathrin-coated pits and vesicles without other pleiotropic effects (Damke *et al*., 1994, 1995a,b; Baba *et al*., 1995). In the experiments described below, we used several HeLa cell

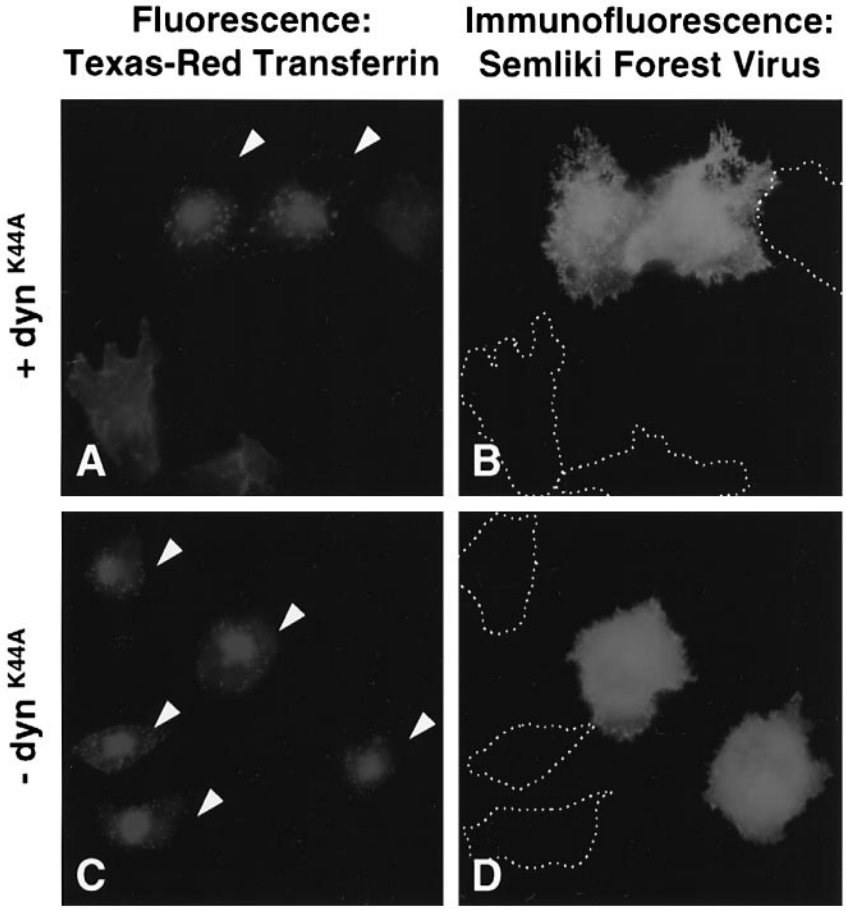

Fig. 1. Productive infection of HeLa cells by SFV coincides with the ability of the cells to internalize Texas Red transferrin through the clathrin-dependent endocytic pathway. HeLa dyn^{K44A} cells, induced to either express (**A**, **B**) or not to express (**C**, **D**) dynamin^{K44A} were incubated with SFV. At 9 h post infection, the cells were processed for immunofluorescence with a polyclonal antibody against the SFV envelope glycoproteins (B, D). Successful infection was scored by the appearance of bright green-labeled cells (light grey on this reproduction). Dotted outlines depict non-infected cells. The ability to internalize transferrin through the clathrin-dependent pathway was established by incubation of the cells with Texas Red transferrin prior to fixation (A, C). Internalization of transferrin results in a punctuate pattern that is characteristic of the endosomal compartment (arrowheads).

lines that inducibly express these mutant forms of dynamin. A similar endocytic block was also obtained using transient transfection of dynamin cDNAs. Infectious events were detected by immunofluorescence microscopy of newly synthesized viral proteins observed in individual infected cells. We used MOIs of <1 to maximize the probability that each infected cell had received only a single viral 'hit', minimizing the risk that a lower-affinity or non-specific entry route had been used for infection. We show here that two enveloped viruses, SFV and Sindbis virus, and the non-enveloped human rhinovirus 14 (HRV 14) depend on the clathrin pathway to enter HeLa cells, whereas the non-enveloped poliovirus does not.

Results

Validation of the single-hit infection assay

As SFV was thought already to use the clathrin-dependent endocytic pathway for entry into cells, we first asked whether blockage of the clathrin endocytic pathway in HeLa cells by expression of mutant forms of dynamin could prevent infection by this virus. We used fluorescence microscopy to assess the ability of a given cell to take up transferrin, and in parallel—using immunofluorescence with antibodies against viral proteins—determined whether the same cell was infected.

Cells active in clathrin-dependent receptor-mediated endocytosis of Texas Red transferrin display a well-established (Ghosh *et al*., 1994) and characteristic intracellular punctuate pattern, which is known to correspond to the endosomal compartment (Figures 1A and 3C, arrowheads). Cells prevented from performing clathrin-mediated endocytosis by expression of the dynamin mutants were devoid of the punctuate staining pattern (Figures 1A and 3A), as expected (Damke *et al*., 1994), with the transferrin remaining mostly at the cell surface. Less than 30% of the HeLa cells expressing either dynamin^{K44A} or dynamin^{ts} (at 38°C, the non-permissive temperature) still support efficient internalization of transferrin, indicating that the clathrin pathway is blocked in most of these cells. The remaining transferrin uptake is a result of non-homogeneous expression of the mutant proteins within the population of cells (Damke *et al*., 1995b). It was therefore necessary to examine individual cells to determine whether the cells that failed to internalize transferrin could be infected by SFV.

We scored each cell for two parameters: appearance of Texas Red-labeled transferrin in the endosomal compart-

Table I. Internalization of transferrin (Tfn) and productive infection by SFV and Sindbis virus

Cell type	µl virus/well	No. of infected cells internalizing Tfn/total no. of infected cells[a]	
		In cells not induced to express dynamin	In cells induced to express dynamin
HeLa dyn^{K44A}	25 Semliki	138/140 (99%)	4/4 (100%)
	50 Semliki	153/163 (94%)	27/28 (95%)
	100 Semliki	123/125 (98%)	19/22 (86%)
	200 Sindbis	105/105 (100%)	94/100 (94%)
HeLa dyn^{ts}	25 Semliki	11/11 (100%)	1/1 (100%)
	50 Semliki	12/12 (100%)	4/4 (100%)
	100 Semliki	35/35 (100%)	0/0 (–)
HeLa dyn^{wt}	25 Semliki	216/217 (100%)	71/71 (100%)
	50 Semliki	354/360 (98%)	281/287 (98%)
	100 Semliki	391/393 (99%)	162/166 (98%)
	200 Sindbis	119/120 (99%)	108/108 (100%)

[a]HeLa cells expressing wild-type or mutant forms of dynamin were incubated with SFV or Sindbis virus and then exposed to Texas Red-labeled transferrin according to the protocol described in Materials and methods. The ability of cells to perform dynamin-dependent, clathrin-mediated endocytosis and to become infected was scored as described in the text.

ment, and appearance of newly synthesized viral envelope glycoproteins. The envelope glycoproteins were detected by immunofluorescence with an anti-spike polyclonal antibody as a generalized staining pattern at 9 h after infection (Figure 1B and D). Essentially all infected cells were active for transferrin endocytosis, whereas cells blocked in transferrin uptake remained uninfected (Table I). Several independent experiments were performed with increasing amounts of SFV using cells expressing either the $dynamin^{K44A}$ or the $dynamin^{ts}$ mutant. These studies showed that HeLa cells blocked for clathrin-mediated endocytosis were 5- to 6-fold less likely to become infected than cells which support normal clathrin-mediated endocytosis (Figure 2). Thus, we have confirmed the proposal that SFV requires clathrin-mediated endocytosis for productive infection of HeLa cells.

Sindbis virus requires clathrin-mediated endocytosis for entry

We next used this immunofluorescent infection assay to determine whether Sindbis virus, which is closely related to SFV, also uses the dynamin-dependent clathrin-mediated pathway as its route of entry into HeLa cells. Bright green anti-envelope glycoprotein staining indicates an infected cell (Figure 3B) and in each case the same cell is also positive for transferrin uptake (Figure 3A, arrowhead). Infection of HeLa cells by Sindbis virus is almost completely restricted to cells that are actively performing clathrin-mediated endocytosis (see Table I). We conclude that Sindbis virus, like its cousin SFV, indeed uses the clathrin-dependent endocytosis pathway to infect HeLa cells, consistent with the observation that exposure to an acidic cellular compartment is required for productive Sindbis virus infection.

It is important to note that the experiments described here were performed with viruses directly obtained from the supernatant of infected cells, without freezing. Virus stocks are often frozen for storage, and unless care is taken this procedure may lower the pH of medium during freezing. We compared frozen and non-frozen stocks, in the presence or absence of buffer (10 mM HEPES pH 7.4), and found that frozen, unbuffered virus stocks enter HeLa cells via a clathrin-independent pathway

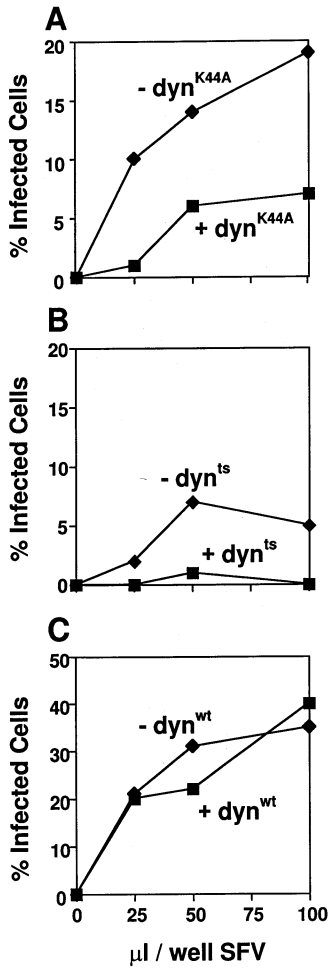

Fig. 2. Decrease in the relative efficiency of SFV infection of HeLa cells correlates with the expression of $dynamin^{K44A}$ (**A**) and $dynamin^{ts}$ (**B**), but not with the expression of $dynamin^{wt}$ (**C**). The data for each point were obtained from four random fields images such as those depicted in Figure 1. At least 350 cells were counted per data point. Each cell line had a different susceptibility to SFV infection, even without expression of ectopic dynamin, presumably reflecting differences in the amount of virus receptor at the cell surface.

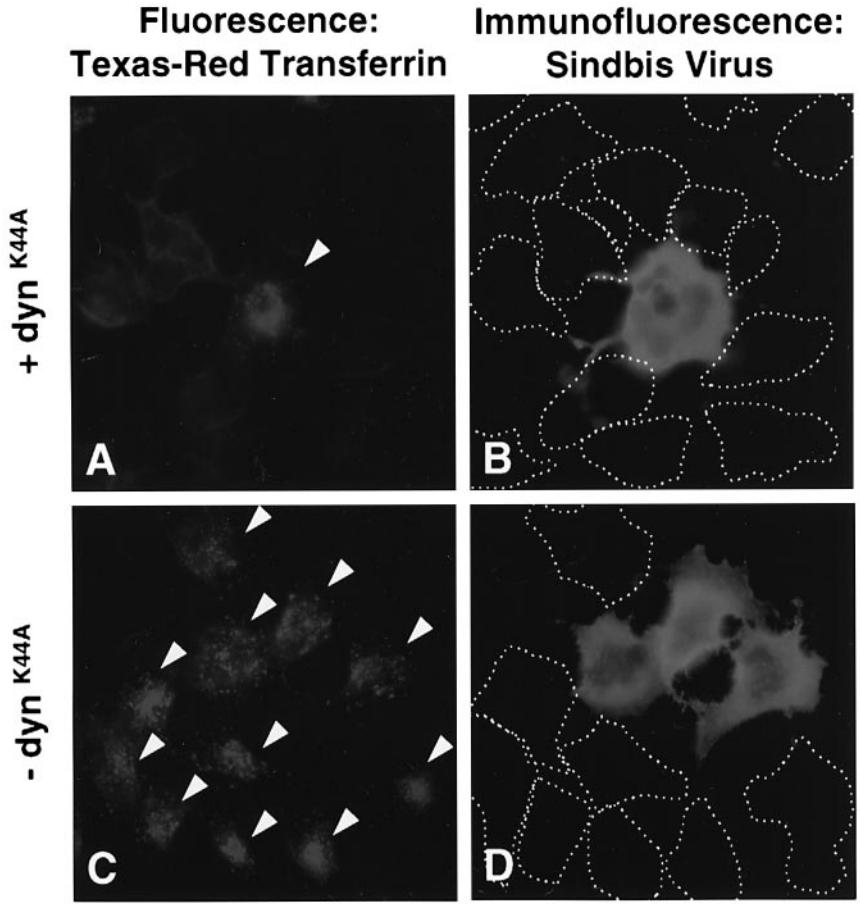

Fig. 3. Productive infection of HeLa cells by Sindbis virus coincides with their ability to internalize Texas Red transferrin. HeLa dyn^{K44A} cells which were induced to express (**A**, **B**) or not to express (**C**, **D**) dynamin^{K44A} were incubated with Sindbis virus. At 8 h post infection the cells were incubated with Texas Red transferrin (A, C) followed by fixation and immunofluorescence with a polyclonal antibody against the envelope glycoproteins (B, D). Successful internalization of transferrin through the clathrin-dependent pathway was scored by the punctuate appearance of the endosomal compartment upon uptake of Texas Red transferrin (arrowheads). Infected cells were identified by their bright green staining (light grey here); dotted outlines indicate non-infected cells.

(data not shown). This observation may go some way towards resolving the conflicting data obtained in other studies regarding the entry pathway of this virus (Fan and Sefton, 1978; Coombs *et al*., 1981; Edwards and Brown, 1981).

Infection by HRV 14 requires clathrin-mediated endocytosis

The entry route of HRV 14 has been a subject of debate for some years (Madshus *et al*., 1987; Perez and Carrasco, 1993). However, we were unable to use the assay described above to address this question for two reasons. First, the HeLa dyn^{wt} and heLa dyn^{K44A} used in these studies are not susceptible to rhinovirus infection; we have determined that lines lack ICAM-1, the rhinovirus receptor (Greve *et al*., 1989; Staunton *et al*., 1989; Tomassini *et al*., 1989). Secondly, infection by human rhinoviruses appears to perturb clathrin-mediated endocytosis; in control experiments using a HeLa cell line that does express ICAM-1 (HeLa H1), we found that cells infected with HRV 14 frequently failed to endocytose transferrin, and some cells that did show clathrin-mediated endocytosis had an altered punctuate pattern. To determine whether infection by HRV 14 requires clathrin-mediated endocytosis, we therefore designed an alternative system in which dynamin expression was monitored directly. This modification allowed us to differentiate between cells that lack endocytic activity due to viral infection and those that lack activity due to the expression of mutant forms of dynamin. The ICAM-1-expressing HeLa H1 line was transiently transfected with plasmids encoding either HA-dynamin^{K44A} or HA-dynamin^{wt}, and individual cells were scored for expression of dynamin (using an antibody that recognizes the HA tag fused to both forms of the protein) as well as for expression of VP3 coat protein (Figure 4). Like SFV and Sindbis virus, HRV 14 shows a strong dependence on clathrin-mediated endocytosis for cell entry: cells expressing mutant, but not wild-type, dynamin were very rarely infected by HRV 14 (Table II).

Poliovirus infection is not dependent on clathrin-mediated endocytosis

Poliovirus, a non-enveloped virus, is another virus for which the route of entry is unclear (Dunnebacke *et al*., 1969; Madshus *et al*., 1984, 1987; Zeichhardt *et al*., 1985; Willingmann *et al*., 1989). Like HRV 14, poliovirus perturbs clathrin-mediated endocytosis; in fact, nearly all cells infected with poliovirus fail to show internalization of transferrin (Figure 5C and D, asterisk). However, the cell lines stably expressing the dynamin variants can be

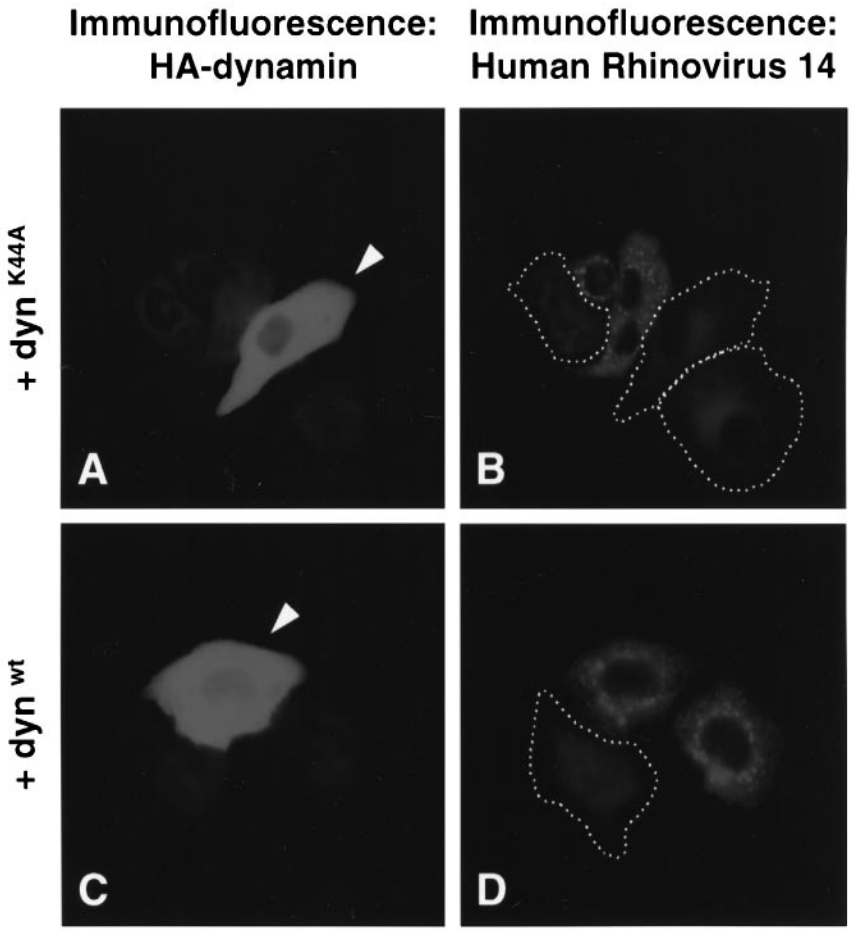

Fig. 4. Productive infection of HeLa H1 cells by HRV 14 is prevented by overexpression of HA-dynamin^{K44A}. HeLa H1 cells were transiently transfected with plasmids encoding HA-tagged dynamin^{K44A} (**A**, **B**) used to block clathrin-dependent endocytosis or HA-tagged dynamin^{wt} (**C**, **D**) used as a negative control. Two days post transfection, the cells were incubated with HRV 14 and analyzed 8 h post infection. Cells expressing dynamin were recognized by the bright green immunofluorescence staining (light grey here) using an FITC-labeled monoclonal antibody specific for the HA-epitope tag located at the N-terminus of dynamin (A, C). Productive HRV 14 infection was scored by the red staining of the cells treated with a monoclonal antibody specific for HRV 14 VP3 coat protein (B, D).

Table II. Internalization of transferrin and productive infection by HRV 14

		No. of infected cells expressing dynamin/ total no. of cells expressing dynamin[a]	
DNA/well[b]	µl virus/well	dyn^{K44A}	dyn^{wt}
1.5 µg	50	5/396 (1%)	108/619 (17%)
3.0 µg	100	11/319 (3%)	115/461 (25%)

[a]HeLa cells transiently expressing wild-type or mutant forms of dynamin were incubated with HRV 14. At 8 h post infection, the cells were processed for immunofluorescence using the FITC-labeled anti-HA antibody (to identify transfected cells) followed by a block step using non-specific mouse antibody, and then the anti-VP3 monoclonal antibody post-labeled with a rhodamine-labeled goat anti-mouse antibody to identify infected cells.
[b]Amount of plasmid DNA used for transient transfection of HeLa H1 cells.

infected with poliovirus, and it was therefore possible to examine the relationship between clathrin-coated vesicle formation and infection in these cell lines. Figure 6 shows that expression of dynamin^{wt} or dynamin^{K44A} has little effect on the overall amount of infection by poliovirus at a variety of MOIs. Since >70% of the cells in this population express the mutant forms of dynamin to a level that blocks transferrin internalization completely, and the maximum infection frequency that can be reached in these cells is ~100% (data not shown), we conclude that poliovirus is not dependent on a clathrin-mediated endocytosis pathway for entry into HeLa cells.

Discussion

We used cells expressing dominant-negative forms of dynamin that block the clathrin-dependent endocytic pathway, but do not prevent fluid phase uptake (Damke *et al*., 1995a), to examine the mode of entry of SFV, Sindbis virus, HRV 14 and poliovirus. Virus entry by routes such as direct fusion at the cell membrane, or micropinocytosis, should not be affected by a disruption of clathrin-mediated endocytosis. We find that SFV, Sindbis virus and HRV 14 infect only cells in which the clathrin endocytic pathway is active. In contrast, poliovirus was not prevented from entering cells in which clathrin-mediated endocytosis is blocked. Thus, SFV, Sindbis virus and HRV 14—but not poliovirus—appear to enter HeLa cells through the clathrin-mediated pathway.

The approach used here allowed us to identify individual

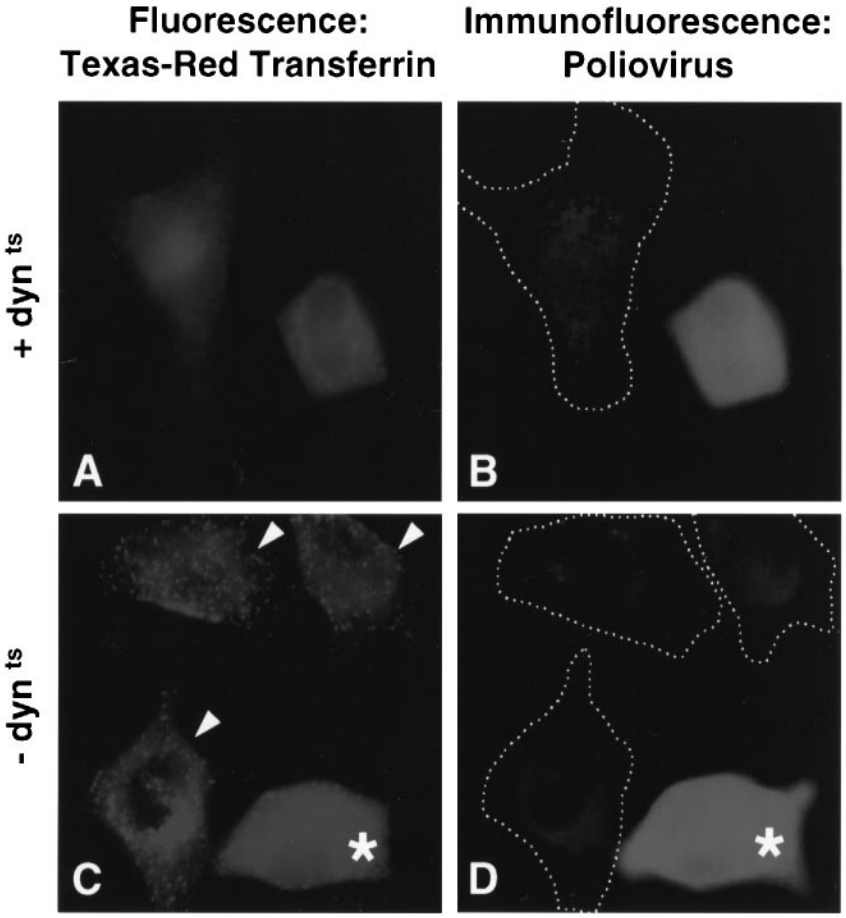

Fig. 5. Infection of HeLa cells with poliovirus is not affected by dynamin^{ts}. HeLa dyn^{ts} cells were induced to either express (**A**, **B**) or not to express (**C**, **D**) dynamin^{ts}. The cells were shifted to 38°C (the non-permissive temperature) and then incubated with poliovirus. At 5 h post infection, the cells were incubated with Texas Red transferrin and then processed for immunofluorescence with a monoclonal antibody against poliovirus VP1 coat protein. Cells performing clathrin-mediated endocytosis showed the characteristic endosomal punctuate pattern [arrowheads in (C)]. Infected cells [asterisks in (C) and (D)] present a marked disruption of clathrin-mediated endocytosis which prevents transferrin uptake to the endosomal compartment. In a parallel experiment (not shown) it was established that, at the non-permissive temperature, >70% of the cells expressing dynamin^{ts} are impaired in the uptake of Texas Red transferrin.

newly infected cells by immunofluorescence microscopy, just a few hours after incubation of the cells with virus. We were therefore able to use low MOIs (<1), representing a concentration of infecting virus similar to those expected *in vivo*. The pathways used for infection in these experiments are likely to be the most efficient routes for cell entry available to the virus, and also the routes most likely to be physiologically relevant.

The results described here for SFV were expected and are in complete agreement with previous observations, indicating that clathrin is important in SFV infection (Doxsey *et al*., 1987). The fact that the introduction of anti-clathrin antibodies into cells causes only a 60% reduction in SFV infection must therefore be due either to incomplete blockade of clathrin-mediated endocytosis by these antibodies, or to the presence of an alternate pathway that is not observed at low MOIs.

Having confirmed the relevance of our assay, we turned our attention to viruses whose modes of entry are less well understood. There are some indications that Sindbis virus is engulfed by the cell during infection: for example, cells newly infected with Sindbis virus are not sensitive to lysis by anti-spike glycoprotein antibodies and complement, suggesting that the viral antigens do not remain on the surface of the cell after infection (Fan and Sefton, 1978). Furthermore, incubation of cells with NH_4Cl, a weak base thought to increase the pH of endosomal compartments, prevented entry of radiolabeled Sindbis virus into the lysosomes of BHK-21 cells (Talbot and Vance, 1982), indicating that the virus requires a fusion step within the endosome for productive infection. Clathrin-coated vesicles are not the only route of endocytic traffic from the plasma membrane to the endosomal compartment, however (Hansen *et al*., 1993), and the conclusion that the endosome is involved in Sindbis virus infection has also been challenged (Edwards and Brown, 1981; Cassell *et al*., 1984). Our study showed a high level of correlation between Sindbis virus infection and dynamin-dependent transferrin uptake, indicating that clathrin-mediated endocytosis is required for infection in HeLa cells. We have also observed that virus stocks that have been frozen without buffering enter HeLa cells through a clathrin-independent pathway, but this entry route is probably not the physiological pathway. Virus stored under these conditions has a different conformation of surface glycoprotein from virus stored in buffer (S.D.Fuller *et al.*, in press). It seems likely, therefore, that Sindbis virus can undergo a conformational change in

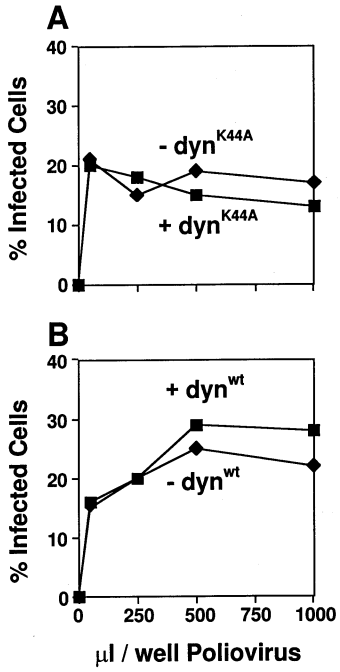

Fig. 6. Infection of HeLa cells by poliovirus is not affected by dynamin^{K44A}. HeLa cells expressing dynamin^{K44A} (**A**) or dynamin^{wt} (**B**) were subjected to poliovirus infection and processed for immunofluorescence according to Figure 5 with the monoclonal antibody against poliovirus VP1 coat protein. At least 1500 cells were scored from three to four random fields for each experimental point.

response to low pH that (unlike the fusion conformation in influenza virus) is stable enough to allow subsequent fusion at the surface of the cell.

In the case of rhinovirus, it is known that successful infection requires an active vacuolar proton-ATPase, since the presence of bafilomycin A1 (a potent and selective inhibitor of the endosomal proton-ATPase) during virus entry prevents infection (Perez and Carrasco, 1993). There is still considerable debate about how rhinoviruses reach the endosomal compartment, however. The role of clathrin-mediated endocytosis has not been studied extensively, but there is some evidence to suggest that this form of endocytosis may not be required for infection (Madshus *et al*., 1987). When cells are transiently depleted of potassium, transferrin uptake by clathrin-coated vesicles can be arrested. This potassium-depletion technique was used to conclude that productive uptake of HRV 2 into Hep2 cells did not require endocytosis from clathrin-coated pits (Madshus *et al*., 1987). Potassium depletion may have many non-specific effects on cells, however, and the mechanism of the block on clathrin-mediated traffic is not known. In our own studies, using HeLa H1 cells transiently expressing mutant dynamin^{K44A}, we found that cells fail to become infected with HRV 14. The transient expression protocol results in inhibition of clathrin-mediated endocytosis that is indistinguishable from the perturbations elicited in cells stably expressing the same dynamin mutants (van der Bliek *et al*., 1993; Damke *et al*., 1994). HeLa cells similarly expressing dynamin^{wt} do become infected. Thus, at least for our combination of virus strain and target cell, clathrin-dependent endocytosis appears to be absolutely required for infection.

How is HRV 14 captured by clathrin-coated vesicles? ICAM-1, the receptor for the major group of human rhinovirus that includes HRV 14, does not contain a recognizable sorting signal for the clathrin pathway, and little is known about its intracellular traffic. Deletion of the cytosolic tail of ICAM-1, or attachment of the ectodomain of ICAM-1 to GPI instead of a transmembrane domain and cytosolic tail, does not affect rhinovirus infection (Staunton *et al*., 1992). For proteins that are directly sorted into clathrin-coated vesicles, such modifications might be expected to prevent internalization. These observations suggest that an unidentified cofactor might be required for efficient capture of the complex between ICAM-1 and HRV 14 into coated pits. Interesttingly, infection by the minor group HRV 2 can require binding to the low-density lipoprotein receptor (Hofer *et al*., 1994). Since it is well established that this receptor internalizes through the clathrin endocytic pathway, it is likely that the same route is used for viral entry.

We have found that infection by poliovirus is not affected upon inhibition of the clathrin-mediated endocytic pathway by expression of dynamin^{K44A}. It is known, however, that this inhibition is rapidly compensated by upregulation of an alternative non-clathrin-dependent pathway of endocytosis (Damke *et al*., 1995a). It is possible that poliovirus can use both endocytic pathways for entry.

We have suggested above that the inhibition of viral infection elicited by the dynamin mutants is a direct consequence of the block in coated pit budding. It is possible, however, that expression of dynamin mutants might prevent infection by affecting (directly or indirectly) other membranous sites known to be involved in later steps of viral replication. This appears unlikely for two reasons. First, poliovirus infection is not affected by the dynamin mutants, even though the viral replication complex requires endoplasmic reticulum membranes for proper function (Bienz *et al*., 1994). Secondly, we have found that Sindbis virus that has been fusogenically activated by freezing (in the absence of added HEPES buffer) and therefore transiently exposed to low pH, can productively infect cells expressing dynamin^{K44A} which are blocked for Texas Red (data not shown). This frozen/low pH-exposed Sindbis virus has a different conformation of surface glycoprotein (S.D.Fuller *et al.*, in press) when compared with virus frozen in the presence of added buffer. Therefore, it is presumed that the virus exposed to low pH can enter cells through the plasma membrane, bypassing the necessity for trafficking to the acidic endosomal compartment. Taken together, these observations indicate that the presence of mutant dynamin does not affect the ability of a target cell to support an infection.

In summary, we have developed a new procedure for studying the involvement of clathrin-mediated endocytosis in viral infection, which uses mutant forms of dynamin to block clathrin-mediated traffic specifically. In this procedure, individual cells are examined for early expression of viral proteins and for endocytotic activity simultaneously, using fluorescence microscopy. Because individual cells, and not bulk populations, are studied, we can use very low MOIs, thus increasing our chance of identifying the physiologically relevant route of infection. We have used this new technique to confirm the require-

ment for clathrin-mediated endocytosis in virus entry by SFV, and we have shown that this pathway is also required for entry by Sindbis virus and HRV 14. The approach of using dynamin to block clathrin-mediated endocytosis has also provided clear evidence that poliovirus does not require the clathrin pathway for infection.

Materials and methods

Cells

HeLa cells stably expressing dynamin^{K44A}, dynamin^{ts} and dynamin^{wt} were kindly supplied by S.Schmid. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal bovine serum (FBS), 2 mM L-glutamine, 400 mg/ml G418, 1× penicillin/streptomycin, 200 ng/ml puromycin and used within 10–12 passages. Expression of dynamin was prevented by inclusion of 1 mg/ml tetracycline in the medium. Cells were induced to overexpress dynamin upon removal of tetracycline. Induction proceeded for 2 days for dynamin^{K44A} and dynamin^{wt} cells, or 3 days for dynamin^{ts} HeLa cells prior to infection (Damke *et al*., 1995a).

These cells were used for the experiments involving SFV, Sindbis virus and poliovirus infections. These lines of HeLa cells lack the ICAM-1 receptor used by HRV 14. Therefore, HeLa H1 cells expressing ICAM-1 and susceptible to HRV 14 infection were used instead. Suspensions of these cells were transiently transfected using the calcium phosphate method with plasmids encoding HA-dynamin^{wt} or HA-dynamin^{K44A}, and used for HRV 14 infection 2 days after transfection.

Viruses and infections

SFV was grown and titered in BHK cells to 2×10^9 PFU/ml. Sindbis virus strain TE12 was grown in BHK cells and titered in chick embryo fibroblasts to 3×10^9 PFU/ml. HRV 14 had a titer of 2×10^8 PFU/ml in HeLa H1 cells. Poliovirus Mahoney type 1 was grown and titered using HeLa S3 cells to 5×10^{11} PFU/ml. The actual number of virus particles per PFU of virus was not determined. The MOI for SFV and Sindbis virus ranged between 0.1 and 0.4; for HRV14, between 0.1 and 0.2; and for poliovirus, between 0.1 and 0.5. These MOIs were deduced from the extent of infection in each experiment.

For virus infections, the target cells were plated at 50% confluence onto cover slips (12 mm diameter) placed in 24-well plates. Frozen virus stocks were thawed and used directly for infections, except for Sindbis virus which was used without freezing. It is possible that these samples might contain some aggregates. All infections were performed in serum-free DMEM containing 1× penicillin/streptomycin, 2 mM glutamine and 1% bovine serum albumin (BSA). Virus dilutions from stock solutions were made with the same medium. Prior to infection, cells were washed twice with serum-free medium containing 1% BSA. The cells were then incubated with 200 µl medium containing the appropriate amount of virus for 1 h at 37°C (33°C for HRV 14) followed by a single wash with serum-free medium containing 1% BSA kept at 37°C. Cells were kept in 1 ml of serum-free medium containing 1% BSA for the duration of the virus growth period (9 h for SFV, 8 h for Sindbis virus, 8 h for HRV 14, and 5 h for poliovirus).

At 20 min before the termination of the virus growth period, 100 µl of 35 µg/ml Texas Red transferrin (Molecular Probes) was added to the medium to label cells for their clathrin-mediated endocytosis phenotype. The experiment was ended by washing the cells twice with ice-cold phosphate-buffered saline containing 1 mM $MgCl_2$, 0.1 mM $CaCl_2$ (PBS^+), followed by fixation with 3% paraformaldehyde for 1 h at room temperature and by 50 mM NH_4Cl for 5 min. Cells were washed once with PBS^+ and processed for immunofluorescence.

Immunofluorescence

To asses for productive infection, the fixed cells were processed for immunofluorescence microscopy by incubation for 1 h at room temperature with the appropriate primary antibodies [1:500 dilution of anti-SFV rabbit serum, 1:100 dilution of anti-Sindbis virus rabbit serum, 1:300 dilution of anti-VP1 (MC17) HRV 14 monoclonal antibody, or 10 µg/ml anti-VP3 (C3) poliovirus monoclonal antibody suspended in PBS^+ containing 0.02% saponin and 1% BSA]. After four washes with PBS^+, the cells were incubated for 45 min at room temperature with FITC-labeled goat anti-rabbit or FITC-labeled goat anti-mouse antibodies. After eight more washes with PBS^+, the coverslips were mounted onto a drop of anti-bleach solution (2 mg/ml *p*-phenylenediamine in 20% PBS^+/80% glycerol) and sealed with nail polish.

Data collection

Images from the SFV, Sindbis virus and poliovirus experiments were recorded for quantitative analysis on Fuji Provia 1600 color slide film. For each experimental point, a minimum of three or four randomly chosen fields were photographed with filter sets for rhodamine or FITC using a Zeiss Axioskop microscope and a ×10 objective lens. In most cases involving SFV and Sindbis virus, at least 300 cells per experimental condition were scored. For poliovirus, at least 1500 cells were scored per experimental point.

Images of the cells acquired in the photographic slides were traced using a camera lucida attached to a dissecting stereo microscope and scored for virus infection and for Texas Red-labeled transferrin uptake. The cells were scored as infected/transferrin uptake-positive, infected/transferrin uptake-negative, uninfected/transferrin uptake-positive or uninfected/transferrin uptake-negative.

The data from the HRV 14 experiments were obtained by direct inspection of the complete coverslips. Cells transiently expressing HA-dynamin^{wt} or HA-dynamin^{K44A} were first identified using a FITC-labeled monoclonal antibody specific for the HA-epitope and then scored for infection by rhodamine-labeled secondary antibody detection of the HRV 14 VP3 coat protein. A minimum of 950 cells were scored per experimental condition.

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