Influenza virus is pleiomorphic, producing both spherical (100-nm-diameter) and filamentous (100-nm by 20-µm) virions. While the spherical virions are known to enter host cells through exploitation of clathrin-mediated endocytosis, the entry pathway for filamentous virions has not been determined, though the existence of an alternative, non-clathrin-, non-caveolin-mediated entry pathway for influenza virus has been known for many years. In this study, we confirm recent results showing that influenza virus utilizes macropinocytosis as an alternate entry pathway. Furthermore, we find that filamentous influenza viruses use macropinocytosis as the primary entry mechanism. Virions enter cells as intact filaments within macropinosomes and are trafficked to the acidic late-endosomal compartment. Low pH triggers a conformational change in the M2 ion channel protein, altering membrane curvature and leading to a fragmentation of the filamentous virions. This fragmentation may enable more-efficient fusion between the viral and endosomal membranes.

Influenza virus is an enveloped virus with a negative-sense RNA genome consisting of eight RNA segments encoding 11 proteins (37). There are three integral membrane proteins, the receptor binding/membrane fusion glycoprotein hemagglutinin (HA), the enzyme neuraminidase (NA), and the proton-selective ion channel (M2). The RNA polymerase complex, consisting of the proteins PB1, PB2, and PA, forms the ribonucleoprotein (RNP) core in conjunction with the nucleocapsid protein (NP). The matrix protein (M1) interacts with the lipid envelope and mediates packaging of the RNP.

Upon contact with the host cell, HA binds to sialic acid moieties on surface-exposed host glycoproteins (12, 23). HA binding triggers clathrin-dependent receptor-mediated endocytosis of the bound virion through the adapter protein Epsin-1 in a dynamin-dependent process (7, 45, 50). Following endocytosis, the virus is trafficked through the endosomal maturation pathway until endosomal acidification triggers the low-pH activation of the HA molecule. Activated HA is then able to mediate the membrane fusion between viral and endosomal membranes (53). Concurrently, the low pH of the endosome activates the proton-selective ion channel activity of the M2 protein, permitting protons to enter the interior of the virus particle. Acidification of the virus interior causes dissociation of the M1 protein from the RNP core, a process which, in conjunction with HA-mediated membrane fusion, is necessary for the release of the viral RNPs and their subsequent import into the nucleus, allowing for viral replication to begin (reviewed in references 25 and 40).

Influenza virus produces pleiomorphic virions that range in size from 100-nm-diameter spherical virions 100 nm in diameter and up to 20 µm in length (1, 5, 8–11, 24, 32). While filament formation is a genetic trait, mapped to the M1 protein (4, 16, 42), additional studies have suggested that the M2 protein may be able to modulate filament formation (22, 29, 43). The M2 protein is a 97-residue homotrimer containing a 24-residue ectodomain, a single transmembrane domain that forms the pore of the ion channel, and a 54-residue cytoplasmic tail that forms a membrane-proximal amphipathic helix (19, 26, 35, 39, 44, 48, 52, 56). Recent work suggests that the M2 cytoplasmic tail is involved in binding to M1 (6) and that this binding recruits M2 to sites of budding (43). Analysis of the M2 amphipathic helix showed that M2 is able to alter membrane curvature in a cholesterol-dependent manner, a property that is essential for the formation of filamentous virions as well as for the efficient release of budding influenza viruses (44).

Previous work on filamentous virions has shown that they contain one copy of the viral genome and possess a specific infectivity similar to that of the spherical forms (36, 42). Intriguingly, freshly isolated influenza virus from the human upper respiratory tract appears to be predominantly filamentous (11, 24), and work with the recent 2009 H1N1 pandemic virus has shown that the virus is able to retain its filamentous morphology upon growth in tissue culture cells (34).

While the entry pathway for spherical virions has been studied extensively, little information is available for the entry pathway utilized by the filamentous forms of influenza virus. The large size of the filamentous virions precludes their entry through canonical clathrin-coated pits (100 nm in diameter); however, an alternate clathrin-independent pathway has already been proposed for spherical virions (46, 51). It was found that 35% of spherical influenza virions were able to undergo entry and membrane fusion without triggering recruitment of the clathrin lattice (46). Similar results were found using inhibitors of clathrin- and caveolar-mediated endocytosis, with which influenza virus entry was able to proceed with near-wild-type (wt) efficiency despite the block in canonical endocytic pathways (46, 51). Recent work utilizing a filamentous strain of influenza virus showed that the virus entered cells as efficiently as the spherical forms though with slightly delayed kinetics that may be attributed to a dependence on an undetermined, dynamin-independent entry pathway (50). This dynamin-independent pathway was recently shown to be capable of mediating the entry of spherical influenza virions when clathrin-mediated endocytosis was inhibited (14). Further examination of
this alternate entry pathway showed that it possesses many of the hallmarks of macropinocytosis (14).

In this study, we confirm that, in addition to the standard clathrin-mediated endocytosis pathway, influenza virus is capable of utilizing macropinocytosis for functional viral entry. We show that spherical influenza virions utilize macropinocytosis as an alternate entry pathway while the filamentous virions enter cells primarily via macropinocytosis. Filamentous influenza virions are engulfed, intact, from the cell surface and traffic to an acidified compartment within the cell in which the low-pH environment allows for M2-mediated alterations in membrane curvature, fragmenting the filamentous virions and allowing for membrane fusion and the release of the viral genome.

**MATERIALS AND METHODS**

**Cells, viruses, and reagents.** Growth of Madin-Darby canine kidney (MDCK) cells, viral stock propagation, and viral infections were as previously described (43). A/California/07/2009 H1N1 virus was kindly provided by the Centers for Disease Control and Prevention (Atlanta, GA). A549 and HEK-293T cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. Antibodies used were αHA (NR3118; BEI, Manassas, VA), αNP (NR4282; BEI), αM2 14C2 (55) and 5C4 (19), and Alexa fluorophore-conjugated secondary antibodies (Invitrogen, Carlsbad, CA). All inhibitors were from Calbiochem (Merck, Darmstadt, Germany) or Sigma (St. Louis, MO) and used at the indicated concentrations. Epsin1-ΔUIM-CFP (7) and Pak1-K299R (49) were purchased from AddGene (Cambridge, MA). Alexa-labeled transferrin, LysoTracker-Red, and alamarBlue cell viability reagents were purchased from Invitrogen and used according to the manufacturer’s instructions. Gold-labeled dextran (10 nm Au-dextran) was purchased from Nanocs (New York, NY). Horseradish peroxidase (HRP) and metal-enhanced 3,3′-diaminobenzidine (DAB) substrate were purchased from Thermo Scientific (Rockford, IL) and used according to the manufacturer’s instructions.

**Entry infections.** Cells were grown on glass coverslips and incubated with virus at the indicated multiplicity of infection (MOI) for 1 h at 4°C to allow for viral attachment. The cells were then washed, warm DMEM was added, and the cells were incubated at 37°C for 8 h. Cells were then fixed in 10% formalin–phosphate-buffered saline (PBS) (EMS, Hatsfield, PA) for 10 min, permeabilized with 0.1% Triton X-100–PBS for 15 min, and stained for NP. Infections in the presence of chemical inhibitors were performed as described above with the cells pretreated with the inhibitor for 1 h at 37°C, followed by 1 h of viral attachment at 4°C and 7 h of incubation at 37°C, both in the presence of the inhibitors. Cells were formalin fixed, permeabilized with 0.05% saponin, and stained for NP. Infections of dominant negative (DN)-expressing cells were performed as described above, utilizing 293T cells 24 h after transfection with the dominant negative construct. 293T transfections were performed with FuGene 6 (Roche, Indianapolis, IN) according to the manufacturer’s instructions. Cells were imaged on a Zeiss (Thornwood, NY) Axiovert 200m microscope using a 40× objective, epifluorescent illumination, and Zeiss AxioVision software.

Quantification of the percentage of infected cells was performed by dividing the number of NP-positive cells by the total number of cells in each field of view over a minimum of 10 random fields of view containing an average of 334 MDCK cells or 795 293T cells, averaged over 3 independent experiments for each condition. Given the high transfection efficiency of 293T cells, all cells in the field of view were counted regardless of transfection state; inclusion of a minimum of infected, untransfected cells in our calculations would only artificially lower our calculated significance, ensuring that our reported significance values represent a minimal value.

For entry in the presence of chemical inhibitors, a minimum of 5 random fields of view were imaged, with a minimum of 300 cells quantified per inhibitor per experiment, with results presented as the averages of two independent experiments. Values were normalized to those of untreated, wt-infected cells, with data displayed as means and standard deviations (SDs). Significance was determined by the Student t test, with a significant result giving a P value of ≤0.05.

**Fluorescent dextran uptake.** Freshly split MDCK cells, with 5 × 10^5 cells per plate, were resuspended in 1 ml DMEM and 10% fetal calf serum at 37°C. Three MOI of the indicated virus was added in the presence of 1 mg/ml dextran-Alexa 488. The samples were gently shaken at 37°C in sealed tubes for 15 min. Dextran uptake was then stopped by the addition of ice-cold PBS. Samples were washed 3 times with cold PBS, fixed with 4% formaldehyde for 15 min, pelleted, and washed 3 times with PBS. Internalized dextran was quantified using a FACScalibur flow cytometer (Becton, Dickinson, Franklin Lakes, NJ). Each sample was assayed in duplicate, and the results are the averages of two independent experiments.

**Immunofluorescence of internalized filamentous virions.** To distinguish between internalized and cell surface-bound virions, cells were grown on glass coverslips, treated as indicated, fixed in 10% formalin–PBS, blocked, and stained with αHA (NR3118) followed by Alexa-488-conjugated donkey anti-goat antibody. The cells were then fixed, washed, permeabilized with 0.05% saponin for 15 min, blocked, and then stained for internal virions with αHA (NR3118) followed by Alexa-594-conjugated donkey anti-goat antibody. Images were collected on a Zeiss LSM5 Pascal confocal microscope. Settings were optimized to eliminate cross-talk between detection channels. Three-dimensional (3D) optical sectioning was performed during imaging, and two-dimensional (2D) maximal intensity projections were generated using AIM software. Postimaging manipulation was performed in Photoshop (Adobe, San Jose, CA) and was limited to image cropping and equal adjustments of image levels.

**Electron microscopy.** Thin-section transmission electron microscopy (TEM) and imaging of viral supernatant and liposome preparations were performed as previously described (44), with images collected on a JEOL 1230 electron microscope (JEOL, Tokyo, Japan) at 100 keV. HRP uptake by A549 cells was performed essentially as described previously (20). Cell-associated HRP was visualized by incubating monolayers with metal-enhanced DAB substrate for 5 min according to the manufacturer’s directions. Prior to use, the dextran-colloidal gold conjugate was concentrated approximately 10-fold by ultracentrifugation. Monolayers of A549 cells were incubated with virus and dextran-gold for 1 h at 4°C, and then the samples were shifted to 37°C for 20 min. Cells were washed 4 times with PBS and processed for electron microscopy as described previously (44). Images were captured using a Gatan 831 digital camera (Pleasanton, CA). For MAb-14C2 and MAb-5C4 treatments, the antibody was first purified using the Melon IgG purification kit (Pierce, Rockford, IL) and used at 15 μg/ml for 1 h at 37°C. The percentage of filamentous virions was calculated following TEM imaging of A/UDorn/72 supernatant. Over 300 virions were imaged, and their lengths were calculated using ImageJ (National Institutes of Health, Bethesda, MD). Virions termed filamentous possessed lengths greater than 250 nm (or a length greater than the estimated diameter of a clathrin-coated pit). Control samples consisted of more than 300 A/WSN/33 virions, of which only 4.9% had lengths greater than or equal to 250 nm. TEM postimaging manipulation was performed in Photoshop (Adobe, San Jose, CA) and was limited to image cropping and equal adjustments of image levels.

**Large unilamellar vesicles.** Large unilamellar vesicles (LUVs) were prepared from a 4:1:2 molar ratio of 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleyl-sn-glycero-3-phospho-(1′-rac-glycerol) (POPG)/cholesterol (Avanti Polar Lipids, Alabaster, AL) or a 4:1 molar ratio of POPC/POPG using 12.5 mM total lipid as described previously (44). Lipid solutions were mixed, dried under argon gas, and lyophilized in a FreezeZone 2.5Plus lyophilizer (Labconco, Kansas City, MO). Lipid films were reconstituted in K buffer (10 mM K2HPO4, 30 mM K2SO4, 5 mM MOPS [morpholinopropanesulfonic acid] [pH 7.4], 40 mM octogluconate) with 100 μg of purified protein where indicated. M2 and M2-helix protein were expressed in HI5 insect cells.
cells from recombinant baculoviruses, and the proteins were purified as previously described (44). Protein was incorporated into LUVs by 15 cycles of freeze-thaw, followed by extrusion 21 times through a 100-nm polycarbonate membrane using an Avanti extruder (Avanti Polar Lipids, Alabaster, AL).

RESULTS

Macropinocytosis functions as an alternate entry pathway for influenza virus. Certain strains of influenza virus, such as A/Udorn/72, are capable of producing filamentous as well as spherical virions. Whereas spherical virions are known to enter host cells by triggering clathrin-mediated endocytosis, filamentous virions are too large to fit in a canonical clathrin-coated pit and their mechanism of entry is not known. Previous work has shown that spherical influenza virus is capable of infecting cells in the absence of clathrin-mediated endocytosis through a serum-dependent induction of macropinocytosis (14). To assess the possible role of macropinocytosis in the entry of filamentous influenza viruses, we inhibited clathrin-mediated endocytosis in conjunction with inhibitors of macropinocytosis and assessed the resulting viral infectivity in the presence of serum by staining for NP expression at 7 h postinfection (h.p.i.). Consistent with previous results, we observed that blockade of clathrin-mediated endocytosis by the inhibitor chlorpromazine (CPZ) had a moderate effect on the ability of influenza virus to infect MDCK cells, while inhibition of endosomal acidification by ammonium chloride (AmCl) completely blocked infection (Fig. 1a) (28, 53). Inhibition of macropinocytosis by the Na"^+//H"^+ pump inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA) or the Pak1 kinase inhibitor IPA3 significantly reduced viral infectivity when used in conjunction with CPZ (Fig. 1a), further confirming macropinocytosis as an alternate entry pathway for influenza virus.

To confirm the efficacy of inhibition of clathrin-mediated endocytosis, MDCK cells were treated with 15 μg/ml CPZ, and a significant decrease in the uptake of fluorescently labeled transferrin was observed, indicating efficient inhibition of clathrin-mediated endocytosis (Fig. 1b). To further evaluate the role of macropinocytosis for influenza virus entry, MDCK cells were treated with CPZ in conjunction with known inhibitors of macropinocytosis and viral infectivity was assessed by staining for NP expression at 7 h.p.i. Similar to the findings of de Vries and coworkers (14), a significant inhibition of influenza virus entry was found with a broad range of macropinocytosis inhibitors, with further significant decreases in infectivity seen when clathrin-mediated endocytosis was also inhibited with CPZ (Fig. 1c) (14). Inhibition of protein kinase C by bisindolylmaleimide significantly inhibited influenza virus infection in the presence or absence of CPZ, while inhibition of PI3 kinase by wortmannin caused a more moderate reduction in infectivity. Inhibition of myosin II by blebbistatin, actin by cytochalasin D, or microtubules by nocodazole prevented infection when clathrin-mediated endocytosis was also inhibited. Additionally, inhibition of the Rho family of proteins, including Rho, Ras, and Cdc42, by the Clostridium botulinum C3 toxin and the Clostridium difficile toxin B or inhibition of Rac1 by the Rac1 inhibitor NSC23766 caused a significant attenuation of viral infection in the presence of CPZ. Inhibition of macropinocytosis by treatment with IPA3 or EIPA attenuated viral infectivity and completely prevented infection by influenza virus when CPZ was also present (Fig. 1a and c). Extraction of membrane cholesterol by methyl-β-cyclohextrin was more effective at inhibiting infectivity in the presence of CPZ, whereas inhibition of dynamin by dynasore significantly attenuated infectivity even when CPZ was not present (Fig. 1c).

Differences in percent inhibition were observed between this study and the work of de Vries et al. (14). However, these differences may be attributable to differences in the cell type (MDCK versus HeLa) and viral strain (A/Udorn/72 versus A/WSN/33) utilized in the experiments, and both sets of data demonstrate the near-complete loss of infectivity seen when both clathrin-mediated endocytosis and macropinocytosis are inhibited. Evaluation of the effects of these inhibitors on cell growth showed that the inhibitors had no adverse effect on cell viability, ensuring that the reduction in viral entry was due to a specific action on the entry pathway and not a general effect on cell viability (Fig. 1d).

Because many chemical inhibitors can exhibit off-target effects, we confirmed the role of macropinocytosis for influenza virus entry through the use of dominant negative (DN) constructs for Epsin1 and Pak1. Epsin1 has been shown to be an essential adaptor protein in the clathrin-mediated endocytosis entry pathway of influenza virus, and deletion of the ubiquitin-interacting motif (UIM) inactivates the protein in a DN fashion (7). Pak1 is a kinase involved in the induction of macropinocytosis, and the kinase dead mutant (K299R) has DN activity (49). DN plasmids were transfected into 293T cells with the low-toxicity transfection reagent Fugene 6 in order to avoid possible reductions in infectivity caused by transfection-mediated induction of interferon. MDCK cells suffer from poor transfection efficiency; to ensure maximal levels of transfection, these experiments were performed in 293T cells. Overexpression of Epsin1-DN/UM (Epsin1-DN) in 293T cells caused a slight reduction in the ability of influenza virus to infect the cells (Fig. 1e), consistent with blocking clathrin-mediated endocytosis. However, overexpression of Pak1-K299R (Pak1-DN) alone caused a significant reduction in viral infectivity, and overexpression of Epsin1-DN and Pak1-DN together caused a much greater loss in viral infectivity (Fig. 1e), suggesting that the filamentous-virion-containing A/Udorn/72 strain of influenza virus may partially utilize macropinocytosis for cellular entry. In contrast, the spherical strain of influenza virus, A/WSN/33, was not affected by Pak1-DN overexpression, though similar losses in infectivity were seen in the combined presence of Epsin1-DN and Pak1-DN, thus indicating that multiple strains of influenza virus can utilize macropinocytosis for viral entry when clathrin-mediated endocytosis is blocked.

Filamentous influenza virions enter cells via macropinocytosis. Given that the virus stocks of A/Udorn/72 are greatly enriched in filamentous virions (43), the data shown in Fig. 1 suggest that filamentous virus as well as spherical virus can use macropinocytosis for entry into cells. Utilization of macropinocytosis as a cellular entry pathway may allow for filamentous virions to enter cells, avoiding the size restriction of clathrin-coated vesicles. To determine if filamentous influenza virions activate macropinocytosis for their primary entry pathway, we treated filamentous influenza virions with the monoclonal antibody MAb-14C2, which we have shown previously to cause fragmentation of the filaments, generating a disperse population of spherical-like virions with only a minor loss of infectivity (43) (Fig. 2c). The treated and untreated virus populations were then used to infect MDCK cells in the presence or absence of the macropinocytosis inhibitor IPA3. IPA3 treatment caused a significant reduction in the infectivity of wild-type (wt) virus but not of MAb-14C2-treated virus (Fig. 2a), suggesting that the filamentous fraction utilizes mac-
FIG 1 Influenza viruses utilize macropinocytosis as an alternate entry pathway. (a) The percentage of infected MDCK cells was determined by an assessment of NP expression following infection with 1 MOI of A/Udorn/72 for 7 h at 37°C in the presence or absence of the indicated inhibitors. Inhibitors were used at the following concentrations: ammonium chloride (AmCl2), 20 mM; chlorpromazine (CPZ), 15 μg/ml; EIPA, 50 μM; IPA3, 10 μM. Values were normalized to the percentage of infected, untreated cells. (b) MDCK cells were pretreated with CPZ at 15 μg/ml for 1 h at 37°C, pulsed with 0.5 mg/ml fluorescently labeled transferrin, and incubated for 1 h at 37°C. Cells were then fixed, and the percentage of transferrin-positive cells was quantified and normalized to the percentage of untreated cells. (c) The percentage of infected MDCK cells was determined as in panel a and normalized to the percentage of infected, CPZ-treated cells. Inhibitors were used at the following concentrations: ammonium chloride (AmCl2), 20 mM; bisindolylmaleimide (bis), 20 μM; blebbistatin (bleb), 100 μM; chlorpromazine (CPZ), 15 μg/ml; cytochalasin D (cytoD), 0.5 μg/ml; dynasore (dyna), 80 μM; EIPA, 50 μM; Clostridium botulinum C3 exotoxin (exoC3), 1 μg/ml; IPA3, 10 μM; methyl-β-cyclodextrin (MβCD), 10 mM; nocardazole (noc), 10 μg/ml; Rac1 inhibitor (rac1ini), 100 μM; Clostridium difficile toxin B (toxB), 300 ng/ml; wortmannin (wort), 1 μM. All single-inhibitor treatments except cytoD showed a significant difference from untreated cells, and all samples with CPZ added (+CPZ) showed a significant difference from CPZ-only-treated cells, as determined by the Student t test (P ≤ 0.05). Note: the data shown in panel a have been included in this full data set to facilitate comparison of the different treatment conditions. (d) MDCK cells were treated with the indicated inhibitors for 4 h at 37°C, alamarBlue cell viability reagent was then added in the presence of the inhibitors, and the cells were incubated for a further 4 h. Cell viability was assessed by quantifying alamarBlue fluorescence. (e) 293T cells were transfected with the indicated DN plasmids or mock-transfected with an empty vector control plasmid, and the percentage of infected cells was determined following 8 h of infection with 1 MOI of A/Udorn/72 or A/WSN/33, as in panel a. Values are means ± SDs. *, significant difference from untreated cells as determined by the Student t test (P ≤ 0.05); **, significant difference from CPZ- or Epsin1-DN-treated cells as determined by the Student t test (P ≤ 0.05).
Filamentous influenza viruses enter cells via macropinocytosis. (a) The percentage of infected MDCK cells was determined following infection with 1 MOI for 8 h. Cells were infected with A/Udorn/72 or with A/Udorn/72 that had been pretreated with MAb-14C2 at 37°C for 1 h before infection. Values were normalized to the percentage of infected, untreated cells. (b) The percentage of infected transfected 293T cells was determined following infection with 1 MOI for 8 h, performed as described above. Cells were infected with either A/Udorn/72, MAb-5C4-treated A/Udorn/72, MAb-14C2-treated A/Udorn/72, A/California/09, or A/WSN/33. Values are means ± SDs. *, significant difference from untreated controls. (c) Morphology of relevant influenza virus strains was determined following infection of MDCK cells with either A/Udorn/72, A/Udorn/72-1a, A/California/09, or A/WSN/33. Cells were infected at 3 MOI for 18 h before fixation, immunofluorescent staining for HA, and imaging by confocal microscopy. Scale bars, 10 μm.

Macropinocytosis for cellular entry whereas the spherical variants are also capable of entering cells via clathrin-mediated endocytosis. Furthermore, the 40 to 50% reduction in infectivity seen when macropinocytosis is inhibited (Fig. 1e and 2a) correlates well with the 31.8% of filamentous virions observed in A/Udorn/72 samples, suggesting that filamentous influenza viruses enter cells primarily via macropinocytosis.

It is important to note that in these experiments, treatment of wt virus with MAB-14C2 caused a slight decrease in infectivity, contrasting with previous results wherein antibody treatment had only minor effects on viral titer (43). One possible explanation for this discrepancy is that the current infections were performed with an MOI of 1 PFU/cell of virus and were scored by evaluating NP expression at 8 h p.i., whereas the previous experiments utilized plaque assays to more-specifically quantify virus titer following low-MOI infections for 48 h. However, to further confirm that the loss of infectivity seen with MAB-14C2-treated virus in the presence of Pak1 inhibition is due to the loss of filamentous virions from the mixed population of viral morphologies and not an effect of antibody treatment, we utilized an alternate approach to generate a solely spherical population of A/Udorn/72 virions. The variant of A/Udorn/72/1a, arose during selection for resistance to the growth restriction of MAb-14C2 and was found to contain a single point mutation in the matrix protein, A41V, which caused a complete loss of filamentous virions while having no effect on viral replication or fitness (42, 54, 55) (Fig. 2c). Thus, by comparing wt virus, the spherical variant 1a, and MAB-14C2-fragmented wt virus, we can assess if the observed reduction in wt virus infectivity by Pak1 inhibition is attributable to the prevention of filamentous virion entry by macropinocytosis while having no effect on the entry of the spherical virions.

Additionally, to further confirm the results of Pak1 inhibition and minimize the possibility of off-target effects from the IPA3 drug treatment, we utilized a DN construct of Pak1 to block macropinocytosis in 293T cells. We observed that the entry of filament-containing wt virus is significantly inhibited when macropinocytosis is blocked by Pak1-K299R expression (Fig. 2b). Treatment of virus with MAB-14C2 caused a loss of filamentous virions and a reduction in Pak1-DN-mediated inhibition of viral infectivity (Fig. 2b). We have shown previously that treatment of wt virus with the isotype control antibody to M2, MAB-5C4, does not cause fragmentation of filamentous virions (43) (Fig. 2c). Similarly, treatment of filamentous virus with MAB-5C4 had no effect on viral infectivity for either wt or Pak1-DN-expressing cells (Fig. 2b), showing that antibody binding to the M2 protein does not account for the loss of infectivity seen with MAB-14C2-treated virions in the presence of Pak1-DN. Infection of Pak1-DN-expressing cells with the spherical variant of A/Udorn/72, 1a, showed no change in infectivity compared to that of wt cells, further supporting the idea that filamentous virions enter cells primarily via macropinocytosis while spherical virions can utilize clathrin-mediated endocytosis or macropinocytosis.

To show that the use of macropinocytosis for filamentous virion entry is not a specific attribute of the laboratory strain of influenza virus, A/Udorn/72, we utilized an additional filamentous strain of influenza virus, the 2009 pandemic H1N1 virus A/California/09 (Fig. 2c). This filamentous virus was also significantly inhibited by Pak1-K299R expression (Fig. 2b), albeit at a slightly lower level than A/Udorn/72. In contrast to the filamentous strains, infection with a laboratory strain that produces only spherical virions, A/WSN/33 (Fig. 2c), showed no loss of infectivity when Pak1-K299R was expressed, similar to the spherical variant A/Udorn/72-1a (Fig. 2b and c). This confirms that spherical virions can enter cells through clathrin-mediated endocytosis or macropinocytosis while filamentous virions appear to enter cells primarily through macropinocytosis.

Influenza virus induces macropinocytosis independently of viral morphology. Because filamentous influenza virions appear capable of utilizing macropinocytosis for their entry, we sought to determine if the induction of macropinocytosis was a consequence of the filamentous morphology or a more intrinsic prop-
Thus, locking the virions in their cellular compartments. At 2 h p.i., venting fusion between the viral and endosomal membranes and, induction of macropinocytosis. 

It has been shown to enhance this fluid phase uptake (14). To assess the activation is fluid phase uptake; viruses that enter cells via macropinocytosis, such as the spherical forms of influenza virus, have been shown to enhance this fluid phase uptake (14). To assess the induction of macropinocytosis by the filamentous forms of influenza virus, A549 cells were briefly incubated with fluorescently labeled dextran and fluid phase uptake was measured by flow cytometry. Each experiment was performed in duplicate, and the results shown are the averages of 2 separate experiments. *, significant difference from untreated cells as determined by the Student t test (P ≤ 0.05).

Macropinocytosis-mediated entry of influenza virus. The data obtained indicate that influenza virus is capable of inducing macropinocytosis and suggest that macropinocytosis is the dominant entry pathway for filamentous virions. To visualize filamentous virions entering cells, we examined thin sections of infected cells in the TEM. At 15 min postinfection (p.i.), influenza virions were visible on the cell surface next to many cellular protrusions resembling membrane blebs or ruffles (Fig. 4a). Filamentous and spherical virions were seen in the process of engulfment by membrane protrusions that appeared to be the initial stage in macropinosome formation (Fig. 4a).

To visualize virions that have been engulfed in macropinosomes, we blocked endosomal acidification with AmCl2, preventing fusion between the viral and endosomal membranes and, thus, locking the virions in their cellular compartments. At 2 h p.i., in the presence of AmCl2, influenza virions were visible in cellular compartments resembling macropinosomes (Fig. 4b). Whereas spherical virions were found in both macropinosomes (Fig. 4b) and clathrin-coated vesicles (Fig. 4e), filamentous virions were seen only in macropinosome-like compartments (Fig. 4b). To confirm that the entering virions were present in macropinosomes, A549 cells were infected with influenza viruses in the presence of the fluid-phase markers Au-dextran and HRP. TEM thin-section analysis showed the presence of filamentous and spherical virions (indicated by red arrows) within labeled structures (indicated by blue arrowheads) resembling macropinosomes (Fig. 4c and d). It should be noted that the observation of oblong virions in Fig. 4b to d may represent filamentous virions that are at oblique angles to the plane of the section and, thus, cannot be visualized in their entirety. Additionally, the viruses appear to be present in enclosed, internalized macropinosome compartments. However, the caveat has to be added that it is not possible to prove that these compartments are not open to the cell surface at a different plane of sectioning. Thus, to further confirm that filamentous influenza viruses enter cells via macropinocytosis, we utilized an alternate immunofluorescence-based approach for visualizing internalized virions.

Macropinocytosis-mediated entry of influenza virus. The data obtained indicate that influenza virus is capable of inducing macropinocytosis and suggest that macropinocytosis is the dominant entry pathway for filamentous virions. To visualize filamentous virions entering cells, we examined thin sections of infected cells in the TEM. At 15 min postinfection (p.i.), influenza virions were visible on the cell surface next to many cellular protrusions resembling membrane blebs or ruffles (Fig. 4a). Filamentous and spherical virions were seen in the process of engulfment by membrane protrusions that appeared to be the initial stage in macropinosome formation (Fig. 4a).

To visualize virions that have been engulfed in macropinosomes, we blocked endosomal acidification with AmCl2, preventing fusion between the viral and endosomal membranes and, thus, locking the virions in their cellular compartments. At 2 h p.i., in the presence of AmCl2, influenza virions were visible in cellular compartments resembling macropinosomes (Fig. 4b). Whereas spherical virions were found in both macropinosomes (Fig. 4b) and clathrin-coated vesicles (Fig. 4e), filamentous virions were seen only in macropinosome-like compartments (Fig. 4b). To confirm that the entering virions were present in macropinosomes, A549 cells were infected with influenza viruses in the presence of the fluid-phase markers Au-dextran and HRP. TEM thin-section analysis showed the presence of filamentous and spherical virions (indicated by red arrows) within labeled structures (indicated by blue arrowheads) resembling macropinosomes (Fig. 4c and d). It should be noted that the observation of oblong virions in Fig. 4b to d may represent filamentous virions that are at oblique angles to the plane of the section and, thus, cannot be visualized in their entirety. Additionally, the viruses appear to be present in enclosed, internalized macropinosome compartments. However, the caveat has to be added that it is not possible to prove that these compartments are not open to the cell surface at a different plane of sectioning. Thus, to further confirm that filamentous influenza viruses enter cells via macropinocytosis, we utilized an alternate immunofluorescence-based approach for visualizing internalized virions.

Filamentous influenza virions fragment during endosomal acidification. In order to visualize the fate of filamentous virions upon host cell contact, we devised an imaging method to discriminate between intracellular and extracellular virions. Unfortunately, live-cell imaging of virus entry is not possible, as influenza virus does not tolerate the incorporation of tagged proteins into an infectious virus and the filamentous virions cannot be labeled with lipophilic dyes since their length and fragility prevent purification and removal of the unincorporated dye. Thus, to perform our immunofluorescence-based assay, we infected cells for various amounts of time with a high MOI of A/Udorn/72. Cells were then fixed, and the remaining surface-bound noninternalized virus was stained with an excess of αHA antibody followed by an excess of fluorophore-tagged secondary antibody. Antibodies were then fixed to the surface-exposed virus to prevent diffusion, and the cells were gently permeabilized with saponin. Internalized virus was then stained using a limiting amount of the same αHA antibody and the same secondary antibody with a different fluorophore. With this technique, surface-exposed virions can be detected with both secondary antibodies while internalized virions stain only with the postpermeabilization antibody. The specificity of this staining approach is validated by the observation that when virus is allowed to bind the cell surface at 4°C, internalized virions are never detected (Fig. 5a, 0 min). It is important to note that in each field of view, only a small number of filaments are observed (Fig. 5a), due to the low efficiency of the entry and staining methodology and the great fragility of the filaments. Nonetheless, filamentous virions are consistently observed, and additional examples are presented in Fig. 5b.

By using this imaging method, we were able to visualize filamentous virions attached to the cell surface before entry had begun (Fig. 5a). From 15 to 30 min p.i., we observed the presence of filamentous virions within the cell (Fig. 5a and b). The presence of internally stained filaments confirms the observations made in Fig. 4, showing that filamentous virions are able to enter the cell without requiring fragmentation. Interestingly, by 60 min p.i., no filamentous virions were visible within the cell; however, an increased number of spherical particles were observed (Fig. 5a), suggesting that the filamentous virions may fragment into spherical-like particles during macropinosome maturation.

We next investigated the trafficking of filamentous virions to determine if the loss of the filamentous form can be correlated with passage through a specific cellular compartment. We ob-
served that at early times p.i., entering influenza viruses, both filamentous as well as some spherical virions, colocalize with fluorescent dextran, a marker of the macropinosome (Fig. 6a). By 60 min p.i., filamentous virions were no longer evident within the cell and no colocalization was observed between the remaining spherical-like particles and the macropinosome compartment (Fig. 6a), indicating that by the time the filamentous form is lost, the viral entry pathway has diverged from the macropinosome.

By utilizing the fluorescent probe LysoTracker as a marker of the late-endosomal and lysosomal compartments, no colocalization was observed between internalized influenza virus filaments and acidified cellular compartments at 15 min p.i. (Fig. 6b). However, by 30 min p.i., one end of the viral filaments was seen to colocalize with the acidified compartment (Fig. 6b). This process appeared to continue until 60 min p.i., when the filamentous virions were no longer present and the remaining spherical virions and spherical-like particles extensively colocalized with an acidified compartment, suggesting that filamentous virions may fragment into spherical-like particles upon encountering the low pH of the late endosome (Fig. 6b). While many of the colocalizing particles are likely spherical virions that have entered via clathrin-mediated endocytosis, it is suggested that the disappearance of
filamentous virions coincides with the colocalization of spherical-like particles and the acidified compartment. Additionally, when endosomal acidification was inhibited by AmCl2, internalized filamentous virions were evident at 2 h p.i. (Fig. 4b), whereas when endosomal acidification proceeded normally, all filamentous virions were lost from within the cell by 1 h p.i. (Fig. 6b), suggesting that low pH within the late endosome may trigger the fragmentation of influenza virus filaments.

Low-pH activation of the M2 protein alters membrane curvature, fragmenting filamentous influenza virions. To assess the consequences of influenza virus filaments encountering the low pH of the endosome, we treated virus-infected cells with low-pH medium and observed the consequences to viral morphology. Incubation of budding viruses with pH 5.5 medium led to a rapid loss of filamentous virus from the cell surface (Fig. 7a). Similarly, treatment of purified filamentous virions with pH 5.5 medium caused a rapid fragmentation of viral filaments and the formation of many disperse, spherical-like virions visible by TEM (Fig. 7b and c).

As the M2 protein is known to conduct protons following low-pH activation, we sought to test if the low-pH fragmentation of filamentous virions was specifically attributable to the M2 protein. Inhibition of M2 ion channel activity by treatment with the M2 channel blocking drug amantadine was sufficient to block the fragmentation of filamentous virions upon exposure to low pH (Fig. 7a and b).

We have shown previously that filamentous virions can also be fragmented by treatment with the M2 ectodomain MAb-14C2 (43) and that the M2 protein is capable of altering membrane curvature, suggesting that M2-mediated alterations in membrane curvature may be responsible for the observed fragmentation of...
filamentous virions (44). To address the possibility that the low-pH fragmentation of filamentous virions is also mediated by M2-dependent alterations in membrane curvature, we reconstituted M2 protein into LUVs. As has been seen previously, M2 is able to alter membrane curvature, affecting LUV morphology in a manner that is dependent on the presence of an intact amphipathic helix, as LUVs reconstituted with the M2-helix mutant protein (a protein that contains 5 single amino acid mutations in the M2 amphipathic helix) do not possess altered membrane curvature (Fig. 8) (44). Incubation of M2-containing LUVs with MAb-14C2 results in further alteration of membrane curvature and the generation of many small, budding-type vesicles, whereas treatment with the isotype control antibody MAb-5C4 had no effect on LUV morphology (Fig. 8). Similarly, low-pH treatment of M2-containing LUVs resulted in further alteration of membrane curvature and the creation of many small, budding-type vesicles, an effect that was specifically attributable to M2 channel activation, as low-pH-induced alterations in membrane curvature were blocked by treatment with the M2 ion channel blocker amantadine (Fig. 8). Furthermore, no alterations in membrane curvature or in LUV morphology were seen when control LUVs, made without M2 protein, were exposed to low-pH medium (Fig. 8). This suggests that MAb-14C2 and low-pH treatments cause similar conformational changes in the M2 protein, affecting how the M2 protein interacts with the membrane and resulting in further alteration of membrane curvature. Thus, the low pH of the late endosome may cause a conformational change in the M2 protein, altering membrane curvature through the membrane-proximal amphipathic helix and resulting in the fragmentation of filamentous virions.

DISCUSSION

Influenza virus is known to trigger clathrin-mediated endocytosis to facilitate entry into host cells. Interestingly, influenza virus remains capable of infecting host cells, with little to no loss of infectivity, even when clathrin-mediated endocytosis is blocked. Recent work has shown that influenza virus is one of a growing list of viruses that are capable of inducing and utilizing macropinocytosis for cellular entry. However, experiments assessing the entry pathways for influenza virus have been performed predominantly on spherical strains, and much less is known about the entry of filamentous forms of influenza virus. Additionally, it is completely unclear how a 100-nm by 1- to 50-μm filament would be able to fit into a standard clathrin-coated pit. In examining the entry of the filamentous form of influenza virus, we have validated previous results showing that influenza virus can utilize macropinocytosis as an alternate entry pathway (Fig. 1a, c, and e). While the sper-
ical forms of influenza virus appear equally capable of utilizing clathrin-mediated endocytosis and macropinocytosis for their entry, we observed that the filamentous forms of influenza virus enter cells primarily via macropinocytosis (Fig. 2). It is likely that the induction of macropinocytosis by filamentous influenza virions is serum dependent, as has been shown for spherical influenza virions (14), as the two morphological forms appear to trigger macropinocytosis similarly (Fig. 3 and 4).

Our data allow us to postulate a model for the entry of filamentous influenza virions and that is that filamentous virions enter cells as intact filaments through the induction of macropinocytosis (Fig. 4 to 6). It is not clear how macropinocytosis is specifically induced upon virus binding; however, both filamentous and spherical virions are capable of triggering the response (Fig. 3). Intriguingly, recent work has shown that influenza virus is capable of binding to, and triggering the activation of, multiple host receptor tyrosine kinases (RTKs) on the cell surface (15). It is likely that this triggering is caused by RTK binding by the influenza virus receptor binding protein HA. It has been shown that the activation of certain RTKs may trigger the specific induction of clathrin-mediated endocytosis, as reviewed in reference 2, while the activation of other RTKs is known to trigger actin reorganization and the induction of macropinocytosis through the Rab5 GTPase (3, 18, 27). Hunt and colleagues (21) have shown that the activation of the RTK Axl specifically enhances the macropinocytotic uptake of the filamentous Ebola virus (21). Additionally, it has been shown that clathrin-independent entry of influenza virus requires Ras-PI3K signaling, a pathway that can be activated by certain RTKs and can signal the activation of macropinocytosis (17). Work by several groups has also shown that the entry pathway utilized by spherical forms of influenza virus is cell type dependent, with certain cells favoring a macropinocytosis-like pathway while others utilize the more typical clathrin-mediated endocytosis (13, 14, 50). It is possible that the distribution of different RTKs on the different cell surfaces allows for spherical virions to preferentially use one entry pathway or the other. In contrast, our results show that filamentous virions enter cells primarily through macropinocytosis (Fig. 2), in which case it is possible that filamentous virions may be less capable of infecting certain cell types, such as those with a low abundance of macropinocytosis-triggering RTKs on their cell surface, though this remains to be determined.

Our results suggest that the induction of macropinocytosis by filamentous virions causes a rearrangement of the host cell, leading to the formation of membrane protrusions that can be seen engulfing the filamentous virions (Fig. 4). The engulfed filamentous virions are then internalized into the cell within the macropinosome (Fig. 4, 5, and 6a), where they are then trafficked to an acidified cellular compartment (Fig. 6b). The exact trafficking pathway has not been determined in this study; however, it is likely that the macropinosomes fuses with the endolysosomal system, as has been shown previously for Ebola virus (47). Endosomal acidification then exposes the filamentous virion to a low-pH environment, causing fragmentation into numerous spherical-like particles (Fig. 5 to 7). While it was not possible to show directly pH-dependent fragmentation of the filamentous virions within the late endosome, we observed that by the time influenza virions colocalized with an acidified compartment, no filamentous virions remained and only spherical-like particles were visible (Fig. 5 and 6). Furthermore, the direct exposure of filamentous virions to low pH in vitro caused a rapid fragmentation of the virions into spherical-like particles (Fig. 7), suggesting that filamentous virions fragment when exposed to the low-pH environment of the late endosome.

The low-pH fragmentation of filamentous virions is similar to the 14C2-M2 antibody-mediated fragmentation we have reported previously (43). Antibody-mediated fragmentation of filamentous virions appears to be due to the induction of a conformation change in the M2 protein, leading to alterations in membrane curvature (44). Similarly, we observed that low-pH treatment of M2-containing LUVs causes alterations in membrane curvature that can be attributed specifically to activation of the M2 ion channel (Fig. 8). It has been reported that exposure to low pH triggers the opening of the M2 proton channel, causing conformation changes in the protein and movement of the membrane-proximal amphipathic helix (52). This suggests that low-pH and MAb-14C2 treatments may cause similar conformation changes in the M2 protein that are transmitted through the transmembrane domain to the amphipathic helix, resulting in alterations of membrane curvature. Thus, the exposure of filamentous virions to the low pH of the late endosome would cause an opening of the M2 proton channel which is necessary for acidification of the virion core and release of the viral genome. M2 channel activation would also cause a conformational change in the M2 protein, altering viral membrane curvature and leading to fragmentation of the filamentous virion into spherical-like particles. The low-pH environment would also trigger HA-mediated fusion between the viral and endosomal membranes. It is intriguing to speculate that the fusion between multiple, smaller, spherical virions may be more efficient than the fusion between the endosomal membrane and a single filamentous virion, though the energetics of these reactions remain to be determined.

Our results suggest that filamentous influenza virus utilizes macropinocytosis as its cellular entry pathway similarly to other filamentous viruses, such as Ebola virus (33, 41, 47). This is especially interesting given the observation that primary human isolates of influenza virus, as well as the recent 2009 pandemic strain, appear to be predominantly filamentous (11, 24, 34), suggesting that macropinocytosis may be the dominant entry pathway for most strains of influenza virus. As a result, pharmaceutical inhibitors of virally induced macropinocytosis may prove to be a valuable antiviral treatment, effective against multiple different viruses, including, among others, influenza virus, Ebola virus, vaccinia virus, and Nipah virus (30, 33, 38, 41, 47), as reviewed in reference 31. Taken together, our results show that the main entry pathway for filamentous influenza virions is macropinocytosis, whereas the spherical forms of influenza virus are capable of entering cells through either macropinocytosis or clathrin-mediated endocytosis. These results provide an example of two different morphological variants of the same virus that are capable of utilizing different entry pathways for productive infections.

ACKNOWLEDGMENTS

We thank members of the Lamb laboratory for helpful discussions and critical reading of the manuscript. The transmission electron microscopy was performed in Northwestern University’s Biological Imaging Facility. This work was supported by research grant R01 AI-20201 (R.A.L.) from the National Institutes of Allergy and Infectious Diseases. J.S.R. was an Associate, G.P.L. is a Specialist, and R.A.L. is an Investigator of the Howard Hughes Medical Institute.
REFERENCES


